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THE THIAMIN CONTENT OF BREAKFAST CEREALS

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Abstract

Breakfast cereals available in Winnipeg stores were analysed for thiamin by the thiochrome method. Oatmeal, whole wheat cereals, and those containing mixtures of many grains are highest in thiamin content. A single serving may provide from 10 to 15% of the recommended daily allowance. Corn, rice, and wheat endosperm cereals are low in thiamin and contribute but slightly to the daily intake. There is little loss on storage. Cooking losses vary but are for the most part less than 20%. The short thiochrome method of Hoffer, Alcock, and Geddes, which is designed for use with flour, does not determine all of the thiamin in breakfast cereals.

The importance of thiamin in the diet has been well established by investigations covering half a century. The Canadian Council of Nutrition recommends a daily intake of 1.8 mgm. for men and 1.5 mgm. for women; the same quantities are recommended by the Committee on Foods and Nutrition of the National Research Council, Washington (3). More recently it has been suggested that somewhat smaller daily allowances may be adequate.

The best common sources of thiamin are yeast, pork, liver, organs and muscles of many animals, nuts, eggs, legumes, whole grains, and most vegetables. Whole grains are specified, since most of the thiamin in them is concentrated in the germ and outer layers. Thus refined cereal products like white flour are not good sources of the vitamin while whole cereal products like whole wheat flour do contain appreciable amounts of it.

Breakfast foods are made from a variety of grains. Probably all of them contain some of the thiamin-rich portions of the seed, although in widely differing proportions. A few of them are actually fortified by the addition of thiamin concentrates such as wheat germ or rice polishings. Consequently it is to be expected that they will vary a great deal in thiamin content, some being relatively good and others relatively poor sources of this vitamin.

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Comparatively few thiamin assays of breakfast cereals are reported in the literature. Booher, Hortzler, and Hewston (2) have compiled the following results in terms of μgm. per 100 gm. of food: cornflakes, trace; oatmeal, 285 to 975; shredded wheat, 219; wheat germ, 1200 to 6600. Slater and Rial (10) in Australia, Jackson and Malone (7) in Canada, and more recently Kitzes and Elvehjem (8, 9) in the United States have also reported such assays. All of these workers used a thiochrome method. The following are typical of the values they found, the results in each case being reported as or calculated by us to μgm. per gm. of food:

	Slater and Rial (10)	Jackson and Malone (7)	Kitzes and Elvehjem (8, 9)
Bran Flakes	3.9	1.38 – 2.44	3.7 – 5.5*
Corn Flakes		0 – 0.13	3.7 – 5.9*
Oatmeal	1.32 – 5.19	6.16 – 7.51	5.8 – 15.0*
Puffed Rice		0	1.6 – 9.4*
Puffed Wheat		0	4.0 – 5.4*
Shredded Wheat		1.83 – 3.24	1.1 – 2.4
Wheat Flakes		1.18 – 2.87	0.5 – 0.8*

Slater and Rial present several analyses but do not identify their materials very well. The American values marked with asterisks represent cereals fortified with thiamin concentrates. These figures suggest that there are marked differences between the thiamin contents of different breakfast cereals and between different samples of the same kind of cereal. Indeed most of these authors state that their results are tentative only and that more such data should be available before comprehensive conclusions are drawn.

Their reports suggest that there is little destruction of thiamin in the cooking of these foods. Slater and Rial (10) found that very little destruction was evident during the household cooking of porridge. Jackson and Malone (7) after testing several cereals requiring cooking stated that cooking caused no appreciable loss. Hanning (4) found the same to be true for rolled oats. She used a rat test rather than the thiochrome method.

In view of these considerations it was deemed necessary to obtain more data on the thiamin content of breakfast foods, particularly those available in this locality. Further information was needed on cooking losses. There did not appear to be any information on whether the thiamin content remained constant or decreased on storage. Yet this might well be a factor of major importance.

Another point of interest is whether a short method of analysis is satisfactory for such material. The usual thiochrome method of Hennessy and Cerecedo (5) is somewhat time consuming. A rapid method has been proposed by Andrews and Nordgren (1). This has been modified by Hoffer,

Alcock, and Geddes (6). Essentially, these rapid methods eliminate digestion with amylase. Since this step is designed to change phosphoryllated thiamin, or cocarboxyllase, to free thiamin, the rapid method does not account for thiamin in this bound form. Hoffer, Alcock, and Geddes found their method satisfactory for wheat flour, which does not contain cocarboxyllase, but found that it was not adequate for wheat germ, which does. Although a short thiochrome method seemed unlikely to prove satisfactory for breakfast foods it was decided to try one of them in this study to ascertain whether this assumption is correct. The method of Hoffer, Alcock, and Geddes was used.

Experimental

Packages of the common breakfast cereals available in Winnipeg were purchased in the retail stores. Samples were taken from them for investigation. Then the packages were sealed and stored at room temperature for a year. Some packages were stored without this preliminary sampling. At the end of the storage time all of the packages were sampled.

Each sample of a prepared cereal—that is one not requiring cooking—was ground and analysed for moisture and thiamin. Samples of those cereals requiring cooking were divided into two portions. One portion was ground and analysed for moisture and thiamin. The other portion was cooked using the directions given on the package. The cooked cereals were also analysed for moisture and thiamin.

Moisture was determined by the vacuum oven method. Thiamin was determined by the thiochrome method of Hennessy and Cerecedo (5), but using a one hour extraction period. Readings were made with a Coleman Model 12 Electronic Photofluorometer. Thiamin was also determined on the uncooked and prepared cereals after storage by the rapid method of Hoffer, Alcock, and Geddes (6).

The experimental data are presented in Tables I and II. Each cereal is identified by its type rather than by its brand name. The numbers refer to different brands. Where several analyses are reported for one material, each analysis is for a different package of the cereal. In Tables I and II as well are figures that show the amount of thiamin in an average serving of each of the cereals. The weight of such a serving was determined by weighing to the nearest gram ordinary served portions. For cooked cereals a figure of 180 gm. was used. This is three-fourths of a cup. The amounts of thiamin in an average serving were calculated for the newly purchased cereal only. In each case thiamin content as determined by the regular long method was used in the calculation.

The effect of storage is shown in Table III. The thiamin contents of the newly purchased and stored cereals were calculated to a moisture-free basis to facilitate their comparison. In a similar way the effect of cooking is shown in Table IV.

TABLE I

THIAMIN CONTENT OF PREPARED CEREALS

	Newly purchased				After storage		
	Moisture, %	Thiamin, μgm./gm.	Average serving, gm.	Thiamin, in average serving, μgm.	Moisture, %	Thiamin (long method), μgm./gm.	Thiamin, (short method), μgm./gm.
Products derived largely from wheat							
Bran Flakes 1	6.52	2.98	50	149	12.28	2.94	0.65
Bran Flakes 2	6.07	1.51	50	76			
					11.76	2.68	1.22
					11.13	2.43	1.34
Bran Flakes 3	5.00	2.46	50	123	12.19	2.38	1.91
Bran Flakes 4	5.48	2.61	50	131	12.88	1.94	
					12.24		1.44
Whole Wheat 1	5.41	1.84	30	55	11.75	1.82	0.41
Whole Wheat 2	5.69	0.63	28	18	9.61	0.73	0.32
Whole Wheat 3	6.48	2.05	18	37	7.03	1.94	0.83
Whole Wheat 4	4.25	3.07	60	184	8.59	3.56	0.64
Whole Wheat 5	4.95	2.73	40	109	9.39	3.44	1.73
Shredded Wheat 1	6.69	2.28	28	64	9.52	2.37	1.72
Shredded Wheat 2	4.81	2.25	28	63	9.11	1.10	1.05
Puffed Wheat 1	5.74	0.09	15	1	8.35	0.14	0.11
Puffed Wheat 2	7.61	0.16	15	3	8.99	0.08	0.04
					7.61	0.25	0.22
Wheat Germ	7.67	27.49	7	192	9.93	23.20	4.98
					10.63	18.37	2.02
Products derived largely from corn							
Corn Flakes 1	5.07	0.24	25	6	6.34	0.30	0.07
Corn Flakes 2	5.17	0.47	25	12	11.59	0.90	0.10
Corn Flakes 3	4.65	0.55	25	14	8.40	0.18	0.13
Corn Flakes 4	5.43	0.32	25	8	11.81		0.29
					11.17	0.14	0.07
					11.33	0.33	0.16
Puffed Corn 1	6.84	0.16	15	3	11.20	0.17	0.09
Puffed Corn 2	7.22	0.35	15	5	4.87	0.09	0.21
Products derived largely from rice							
Puffed Rice 1	7.48	0.23	15	4			
Puffed Rice 2	6.54	0.46	15	7	11.40	0.15	0.29
Puffed Rice 3	5.68	0.20	30	6	12.02	0.27	0.21
					11.55		0.09
Products not otherwise classified							
Puffed Wheat and Rice	7.46	0.18	15	3	12.12	0.28	0.11
Wheat and Oat Flakes	5.30	3.94	60	236	9.62	2.55	1.88
Mixed Cereals	5.14	2.07	25	52	9.27	1.94	0.12

TABLE II

THIAMIN CONTENT OF COOKED CEREALS

	Newly purchased			After storage		
	Moisture, %	Thiamin, μgm./gm.	Thiamin in average serving (180 gm.), μgm.	Moisture, %	Thiamin (long method), μgm./gm.	Thiamin (short method), μgm./gm.
Products derived from wheat						
Whole Wheat, uncooked	12.68	4.07				
cooked	84.23	0.764	138			
Wheat Farina 1, uncooked	9.25	0.66		12.48	0.35	0.42
cooked	82.32	0.119	21	87.16	0.153	
Wheat Farina 2, uncooked	8.72	0.48		11.67	0.41	0.25
cooked	80.77	0.092	17	87.62	0.092	
uncooked				13.56	0.93	0.66
cooked				87.77	0.238	
Products derived largely from oats						
Rolled Oats 1, uncooked	7.28	6.59		11.28	3.84	
cooked—1 step	84.97	1.038	187	77.93	0.818	
cooked—2 steps	84.61	1.028	185			
Rolled Oats 2, uncooked	8.00	3.98		7.54	3.89	0.24
cooked	80.42	1.519	273	77.90	0.755	
Rolled Oats 3, uncooked	7.85	4.94		6.08	5.56	2.38
cooked	82.34	1.190	214	84.33	0.902	
uncooked				8.48	5.74	1.19
				87.56	1.043	
Products derived largely from barley						
Barley Farina, uncooked	9.27	1.77		14.46	1.77	1.55
cooked	85.04	0.260	47	91.56	0.118	
Products not otherwise classified						
Mixed Cereals 1, uncooked	7.77	4.52		8.90	4.46	2.32
cooked	72.03	1.107	199	81.50	0.890	
Mixed Cereals 2, uncooked	7.92	13.43		13.54	5.49	2.50
cooked	92.28	0.934	168	91.26	0.498	
Mixed Cereals 3, uncooked	8.14	4.28		6.00	3.23	0.42
cooked	70.72	1.277	230	77.63	0.744	
Mixed Cereals 4, uncooked	8.42	4.38		9.60	4.39	3.18
cooked	74.06	1.009	182	84.50	0.448	
Mixed Cereals 5, uncooked	7.95	5.50		7.93	3.14	4.92
cooked	77.88	0.893	161	78.52	0.647	
Mixed Cereals 6, uncooked	9.07	3.80		10.35	1.82	2.51
cooked	81.20	0.637	95	82.71	0.363	
Mixed Cereals 7, uncooked	9.37	4.67		11.33	3.44	3.98
cooked	76.93	0.858	174	36.87	0.729	

TABLE III

THIAMIN CONTENT OF STORED CEREALS

	Thiamin (dry basis), μgm./gm.	
	Newly purchased	After storage
Prepared cereals		
Bran Flakes 1	3.19	3.35
Bran Flakes 3	2.59	2.71
Bran Flakes 4	2.76	2.23
Whole Wheat 1	1.95	2.06
Whole Wheat 2	0.67	0.81
Whole Wheat 3	2.19	2.09
Whole Wheat 4	3.21	3.89
Whole Wheat 5	2.87	3.80
Shredded Wheat 1	2.44	2.62
Shredded Wheat 2	2.36	1.21
Puffed Wheat 1	0.10	0.15
Puffed Wheat 2	0.17	0.09
Wheat Germ	29.77	25.76
Corn Flakes 1	0.25	0.32
Corn Flakes 2	0.50	1.02
Corn Flakes 3	0.58	0.20
Puffed Corn 1	0.17	0.19
Puffed Corn 2	0.38	0.09
Puffed Rice 2	0.49	0.17
Puffed Rice 3	0.21	0.31
Puffed Wheat and Rice	0.19	0.32
Wheat and Oat Flakes	4.16	2.82
Mixed Cereals	2.18	2.14
Cereals requiring cooking		
Wheat Farina 1	0.73	0.40
Wheat Farina 2	0.53	0.46
Rolled Oats 1	7.11	4.33
Rolled Oats 2	4.33	4.21
Rolled Oats 3	5.36	5.92
Barley Farina	1.95	2.07
Mixed Cereals 1	4.90	4.90
Mixed Cereals 2	14.59	6.35
Mixed Cereals 3	4.66	3.44
Mixed Cereals 4	4.78	4.86
Mixed Cereals 5	5.98	3.41
Mixed Cereals 6	4.18	2.03
Mixed Cereals 7	5.15	3.88

Discussion

A comparison of the results obtained by the methods of Hennessy and Cerecedo (5) and of Hoffer, Alcock, and Geddes (6) show that in general the short method gives lower results than the long one. As pointed out previously this was expected since the shorter method does not account for the thiamin in cocarboxyllase. Accordingly the method of Hoffer, Alcock, and Geddes cannot be used for substances as diverse in composition as breakfast cereals,

TABLE IV

THIAMIN LOSS IN COOKING BREAKFAST CEREALS

	Thiamin (dry basis), μgm./gm.			Thiamin lost, %
	Uncooked cereal	Cooked cereal	Cooking loss	
Whole Wheat	4.66	4.84		
Wheat Farina 1	0.73	0.67	0.04	5.5
Wheat Farina 1, stored	0.40	1.19		
Wheat Farina 2	0.53	0.48	0.05	9.5
Wheat Farina 2, stored	0.46	0.74		
	1.08	1.95		
Rolled Oats 1, cooked—1 step	7.11	6.91	0.20	3.6
Rolled Oats 1, cooked—2 steps	7.11	6.68	0.43	6.8
Rolled Oats 1, stored	4.33	3.71	0.62	14.3
Rolled Oats 2	4.33	7.76		
Rolled Oats 2, stored	4.21	3.42	0.79	18.8
Rolled Oats 3	5.36	6.74		
Rolled Oats 3, stored	5.92	5.76	0.16	2.7
	6.27	8.38		
Barley Farina	1.95	1.74	0.21	10.8
Barley Farina, stored	2.07	1.40	0.67	32.4
Mixed Cereals 1	4.90	3.96	0.94	19.2
Mixed Cereals 1, stored	4.90	4.81	0.09	1.8
Mixed Cereals 2	14.59	12.10	2.49	17.7
Mixed Cereals 2, stored	6.35	5.70	0.65	10.2
Mixed Cereals 3	4.66	4.36	0.30	6.4
Mixed Cereals 3, stored	3.44	3.33	0.11	3.2
Mixed Cereals 4	4.78	3.89	0.89	18.6
Mixed Cereals 4, stored	4.86	2.83	2.03	41.8
Mixed Cereals 5	5.98	4.04	1.94	32.4
Mixed Cereals 5, stored	3.41	3.01	0.30	8.9
Mixed Cereals 6	4.18	3.39	0.79	18.9
Mixed Cereals 6, stored	2.03	2.10		
Mixed Cereals 7	5.15	3.72	1.43	27.8
Mixed Cereals 7, stored	3.88	1.15	2.78	70.4

a conclusion that agrees with the observations of these authors. We have, therefore, considered only the results obtained with the method involving digestion with amylase.

The thiamin contents of the cereals analysed are of the same order as those reported by other investigators. Those derived largely from corn and rice are quite low in thiamin. The products derived mainly from wheat endosperm—which we have designated as wheat farina—are likewise not particularly good sources of the vitamin. Those made from whole wheat are much better in this regard: the bran and germ of the wheat contribute substantially to the thiamin in the manufactured cereal. Oatmeal is better than whole wheat. The cereals made from mixtures of several grains appear to be as good and occasionally better sources of thiamin than oatmeal. This is no doubt because they are fortified with wheat bran and germ or rice polishings or other thiamin concentrates. With the exception of those cereals derived mainly from wheat endosperm, breakfast foods that require cooking appear to be better sources of thiamin than prepared ones.

This is substantiated by an examination of the amount of thiamin in an average serving of each of these cereals. One brand of oatmeal contributes between 15 and 16% of the recommended daily allowance in each serving. Oatmeal, whole wheat, and mixed cereals contribute from 10 to 15% per portion. Wheat endosperm, corn, and rice products contribute very little. Cooked cereals contribute more than prepared ones. And this is not because of the difference in size of the portion. The amount of solids in 180 gm. of a cooked cereal is about the same as that in most servings of prepared ones.

Breakfast cereals retain the bulk of their thiamin when stored for considerable periods of time at room temperature. Some show losses but most of these are not very large. The apparent gain in others may represent sampling errors. A possible explanation is that the increase in moisture content of the stored samples makes the extraction of the vitamin easier. It may be concluded that there is little loss of thiamin during storage.

Cooking does not decrease the thiamin content appreciably. Some of the rolled oats and wheat endosperm cereals show apparent gains. It may be that the cooking of these cereals makes possible a more complete extraction of thiamin. This is quite possible with vitreous materials like wheat endosperm. Mixed cereals show the greatest losses, which is not surprising since they often require a longer cooking period. While some individual samples showed a high degree of loss, most of them appeared to have less than 20% of their thiamin destroyed. In half of the cases a loss of less than 10% was found.

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FACTORS AFFECTING THE STORAGE OF DEHYDRATED PORK[1]

By Jesse A. Pearce[2]

Abstract

In an accelerated storage test at 60° C. fat levels of 20 and 30% were observed to have no effect on keeping quality of dehydrated pork. Measurements on dried product stored in paper-bodied containers for one year at temperatures from −17.8° C. to 36.7° C. showed 15.6° C. to be less desirable than 23.9°, 0°, or −17.8° C. Dehydrated pork was stored in tin-plate containers for periods of one year at 23.9° C. and 36.7° C. with little decrease in palatability. No difference in storage life was demonstrated between cured and uncured pork, or, as a result of differences in drying times, moisture content for storage temperature. The product prepared on an atmospheric double-drum drier deteriorated a little more rapidly than vacuum–tray- or tunnel–tray-dried material. The effectiveness of container materials, tin-plate, Reynolds' metal *A*-10, Dewey and Almy *P*-16, and 300 *MST* cellophane, was evaluated and their relative value for dehydrated pork fell in that order.

Introduction

A previous paper (2) described methods of dehydrating pork. Second in importance to the satisfactory preparation of dried foods is the determination of factors affecting storage life. This paper records the effect of methods of preparation, storage temperature, moisture content, and types of packages on the keeping quality of dehydrated pork.

Materials

The materials used in this investigation were those prepared in the previous study (2). Tunnel–tray-dried sow ham (uncured) was used in assessing the majority of the factors believed to affect the storage life of dried pork. Comparisons were made between tunnel–tray-, vacuum–tray-, and atmospheric–drum-dried uncured ham, and between uncured and cured tunnel–tray-dried ham.

Analytical Methods

The methods used for determining moisture content, fat content, peroxide oxygen values, and palatability scores (based on a maximum score of 10) have been described (2). The peroxide values are recorded as ml. 0.002 *N* thiosulphate per gm. of extracted fat.

A preliminary survey had indicated that a fluorescence method may be a useful objective measure of quality of dehydrated pork (3); therefore, this test was used in a portion of the present investigation. The technique used for defatting this product was as follows: the sample was pulverized in a mortar, about 2.5 gm. was defatted by shaking at room temperature with

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[2] *Biochemist, Food Investigations.*

three 25 ml. portions of petrol ether, the ether was drained off through a No. 1 Whatman filter paper, and the remaining solvent allowed to evaporate. The remainder of the procedure was the same as the revised method for dried whole egg powder (5). The results are recorded in Coleman photofluorometer units.

Experimental Methods and Results

The Effect of Fat Content

During preliminary work with dehydrated pork, a product containing about 40% fat was assessed by a taste panel as much less desirable than products with 20 or 30% fat. Therefore in evaluating the effect of fat levels on keeping quality the study was limited to tunnel–tray-dried uncured material with 20 and 30% fat. An accelerated storage trial at 60.0° C. (140° F.) showed that there was little difference in the behaviour of dried pork containing these quantities of fat (Fig. 1). The induction period was slightly reduced

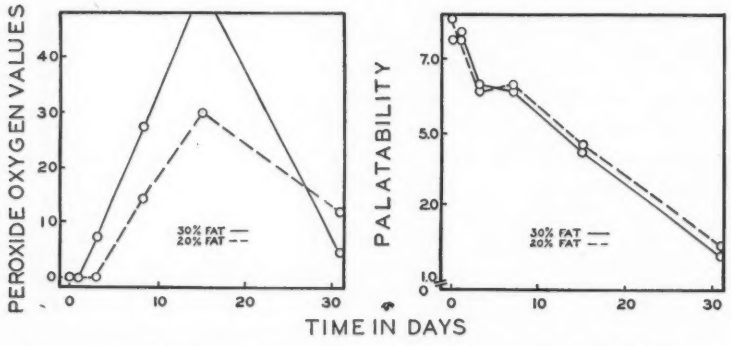

FIG. 1. *The effect of fat content on the quality of dehydrated pork stored at 60.0° C. (140° F.).*

and the maximum value for peroxide oxygen was somewhat greater in the high fat samples. Palatability scores showed that there was a slight preference for the product with the lower fat content: deterioration occurred at about the same rate in both products.

It was concluded that the fat content would have little effect on the rate of deterioration and that studies with material containing 30% fat would be preferable since it is necessary to utilize as much fat as possible and products with this fat content were not distasteful.

The Effect of Storage Conditions

The effect of temperature and humidity was assessed by storing two batches of dehydrated tunnel–tray-dried uncured pork, the one having a moisture content of 6.5% and the other 14%, in paper-bodied containers at −17.8°, 0°, 15.6°, and 36.7° C. (0°, 32°, 60°, and 98° F.) and at relative humidities of 62, 65, 20, and 11% respectively. Several samples were also stored at 23.9° C. (75° F.), relative humidity, 18%. Peroxide oxygen determinations

were made at various times throughout a storage period of one year: palatability was assessed only at the final sampling.

Changes in peroxide value are shown in Fig. 2. At 36.7° C. (98° F.) the peroxide value of both types of dehydrated pork had decreased to zero after

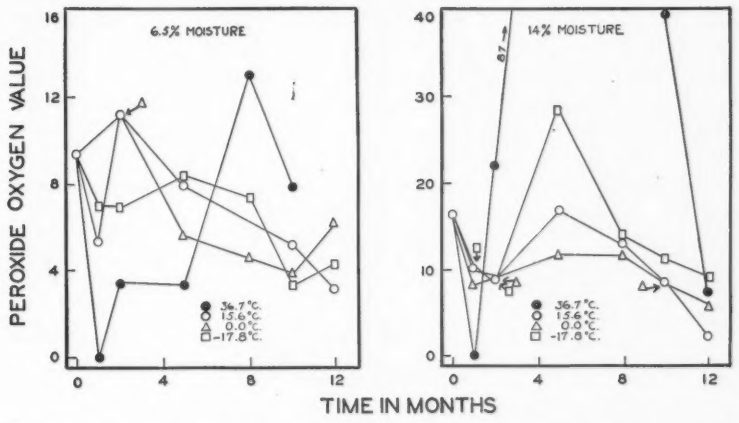

FIG. 2. *Peroxide oxygen changes occurring in dehydrated pork stored at various temperatures in paper-bodied containers.* ● = *36.7° C. (98° F.);* ○ = *15.6° C. (60° F.);* △ = *0.0° C. (32° F.);* □ = *−17.8° C. (0° F.).*

one month; subsequently the value rose to a maximum at about eight months' storage and then decreased to the end of the storage period. The maximum for the high moisture sample was about seven times as great as that for the low moisture sample. At the lower temperatures the trends of the curves are generally similar; a slight decrease during the early part of the storage is evident, followed by an increase and finally a gradual sloping off.

On the basis of palatability measurements (Table I), the samples receiving the lowest scores were those stored at 15.6° C. (60° F.) and 36.7° C. (98° F.), the scores for product at both moisture levels being practically the same. The lower score at the higher temperature was to be expected; however,

TABLE I

PALATABILITY SCORES FOR DEHYDRATED PORK WITH 6.5 AND 14% MOISTURE AFTER STORAGE FOR 12 MONTHS IN PAPER-BODIED CONTAINERS (INITIAL PALATABILITY, 6.6)

Storage temperature		Palatability score	
°C.	°F.	6.5% Moisture	14% Moisture
36.7	98	3.0	2.7
23.9	75	4.1	
15.6	60	3.2	2.9
0	32	4.2	3.9
−17.8	0	5.5	5.2

explanation of the low scores at 15.6° C. (60° F.) would be highly speculative. It may be that this was an optimum temperature for enzyme activity, a type of deterioration possibly differing from that occurring at the higher temperatures. Similar behaviour was noted during the storage of dehydrated mutton (6).

Since the relative humidities in the storage cabinets varied appreciably, it was believed desirable to determine the moisture content of some of the stored samples. With the exception of samples stored below the freezing point, the moisture content was related to the relative humidity (Table II).

TABLE II

CHANGES IN MOISTURE CONTENT OF DEHYDRATED PORK STORED AT VARIOUS TEMPERATURES IN PAPER-BODIED CONTAINERS

Initial moisture, %	Temperature		Relative humidity, %	Moisture content (%) after storage for:	
	°C.	°F.		Two months	Five months
6.5	36.7	98	11	3.6	3.2
	15.6	60	20	5.0	5.4
	0	32	65	8.0	8.9
	−17.8	0	62	8.8	13
14	36.7	98	11	3.6	3.9
	15.6	60	20	5.3	5.9
	0	32	65	9.2	9.4
	−17.8	0	62	13	13

It is evident from these results that, while low temperature storage of dehydrated pork was preferable, the product stored at 23.9° C. (75° F.) was as edible as the product stored for the same period at 0° C. (32° F.). As a result, investigation of other factors was continued only at the higher temperatures.

The Effect of Curing

It has been pointed out that cured ham suffered slightly greater deterioration during the tunnel–tray-drying process, although dried cured ham when reconstituted was usually rated higher than the product from uncured material (2). Peroxide values for dried cured ham and uncured ham stored for one year in tin-plate containers at 23.9° C. (75° F.) and 36.7° C. (98° F.) are given in Fig. 3, A.

The changes in peroxide values were similar for both types of dried product. The samples stored at the lower temperature reached a maximum value after one month's storage, then slowly decreased. Those at the higher temperature decreased rapidly to zero.

Mean palatability scores are given in Table III: none of the factors studied were significant. Since cured ham is more costly, nothing would be gained by using it as the initial material.

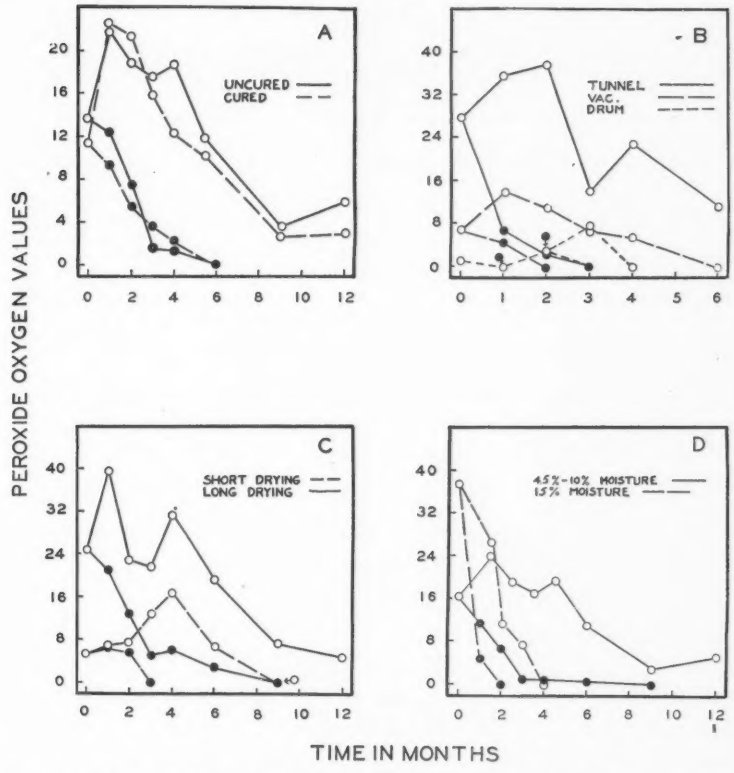

FIG. 3. *Effect of curing (A), method of drying (B), time of drying (C), and moisture content (D) on peroxide oxygen changes in the fat of dehydrated pork.* ● = *36.7° C. (98° F.);* ○ = *23.9° C. (75° F.).*

Effect of Method of Drying

Three drying techniques were investigated during the course of earlier work (2). While tunnel–tray drying produced the best initial product, it was nevertheless believed desirable to measure the keeping quality of uncured pork dehydrated by each of the three methods. The products prepared by different methods of drying had peroxide values increasing in magnitude as follows: atmospheric double-drum, vacuum–tray, and tunnel–tray. It was interesting to note (Fig. 3, B) that the peroxide values again increased to maximum in samples stored at 23.9° C. (75° F.) and then gradually decreased; the maxima were higher for the dried product with greater initial values. At 36.7° C. (98° F.) the peroxide values decreased rapidly to zero.

The significant differences between methods of drying shown in Table IV could for the most part be explained by differences in initial quality; however, pork dried on the double drums deteriorated a little more rapidly during the

TABLE III

MEAN PALATABILITY SCORES FOR TUNNEL–TRAY-DRIED, CURED AND UNCURED PORK, STORED IN TIN-PLATE CONTAINERS FOR ONE YEAR AT 36.7° C. (98° F.) AND 23.9° C. (75° F.)

Variable under investigation	Mean palatability score
Type of material	
Tunnel–tray-dried, cured ham	7.6
Tunnel–tray-dried, uncured ham	7.3
Storage temperature	
36.7° C. (98° F.)	7·4
23.9° C. (75° F.)	7·5
Storage time (months)	
Initial	7.5
1	7.3
2	7.5
3	7.6
4	7.7
6	7.8
9	7.2
12	7.0

last half of the storage period. Therefore the tunnel–tray method, which produces the best initial material, was preferred.

Effect of Drying Period

In the previous experiments it was observed that the dried product with a higher initial peroxide value attained a greater maximum value after a short storage period: this higher initial value was generally indicative of excessive time in the drier. It seemed desirable then to compare the storage life of samples with approximately equal moisture contents but dried for different times and consequently possessing different initial peroxide values.

Batches of about the same moisture content prepared from uncured pork, tunnel–tray-dried under the same conditions for different times, were stored at 23.9° C. (75° F.) and 36.7° C. (98° F.) in tin-plate containers. One batch had a moisture content of 4.6% and an initial peroxide value of 5.4 while the other had 4.8% moisture and a peroxide value of 25.

At the lower temperature, the peroxide oxygen values reached a maximum after a short period in storage, and again the maximum was greater for the product with the greater initial value (Fig. 3, C). At the higher temperature the values decreased gradually to zero, the time required for the decrease being somewhat longer for samples with the higher initial value. Palatability was generally lower for the product with the high peroxide values (Table V), changes during storage being comparable in each case. Again temperature and time were without significant effect. It could be assumed that, except for a general lowering of the palatability values, commencing a storage experiment with dried pork having a high initial peroxide value would not materially alter the rate of decrease of palatability.

TABLE IV

MEAN PALATABILITY SCORES AND ANALYSIS OF VARIANCE FOR PORK DRIED BY DIFFERENT METHODS, STORED IN TIN-PLATE CONTAINERS FOR ONE YEAR AT 36.7° C. (98° F.) AND 23.9° C. (75° F.)

A. Table of means

Variable under study	Mean palatability score
Method of drying	
Tunnel–tray	7.7
Vacuum–tray	7.2
Atmospheric double-drum	6.6
Necessary difference, 5% level	0.53
Storage temperature	
36.7° C. (98° F.)	7.1
23.9° C. (75° F.)	7.2
Storage time (months)	
Initial	6.5
1	7.2
2	7.5
3	7.1
4	7.2
6	6.9
Necessary difference, 5% level	0.37

B. Analysis of variance

Variance attributable to:	D.f.	Mean square
Method of drying	2	2.87**
Temperature	1	0.05
Time	4	0.32*
Method × time	8	0.40**
Residual (error)	13	0.05

* *Exceeds 5% level of statistical significance.*
** *Exceeds 1% level of statistical significance.*

Effect of Moisture Content

In the experiment on storage temperatures the samples employed had different initial moisture contents and these were observed to change during storage. Since low moisture content retards deterioration in stored dried egg powder (7), the storage behaviour of these dried pork samples might likewise be attributed to moisture effects. Therefore, tunnel–tray-dried uncured pork with a range of moisture contents (4.5 to 15%) was stored in tin-plate containers at 23.9° C. (75° F.) and 36.7° C. (98° F.).

The results in Fig. 3, D revealed no difference between the average peroxide values for products with moisture contents from 4.5 to 10%; the behaviour

TABLE V

MEAN PALATABILITY SCORES AND ANALYSIS OF VARIANCE FOR PORK TUNNEL–TRAY-DRIED FOR DIFFERENT PERIODS STORED IN TIN-PLATE CONTAINERS FOR ONE YEAR AT 36.7° C. (98° F.) AND 23.9° C. (75° F.)

A. Table of means

Variable under study	Mean palatability score
Dehydration period	
Short	7.8
Long	7.0
Storage temperature	
36.7° C. (98° F.)	7.3
23.9° C. (75° F.)	7.5
Storage time (months)	
Initial	7.2
1	7.6
2	7.8
3	7.1
4	7.6
6	7.8
9	7.0
12	7.0

B. Analysis of variance

Variance attributable to:	D.f.	Mean square
Dehydration period	1	3.94*
Temperature	1	0.13
Time	6	0.59
Residual (error)	18	0.62

* *Exceeds 5% level of statistical significance.*

was similar to that previously described. The samples with 15% moisture had a higher initial peroxide value and are shown separately. This product, when stored at 23.9° C. (75° F.) behaved unlike any of the samples previously studied; the values decreased to zero without passing through a maximum. This decrease was somewhat slower than for the comparable material at the higher temperature.

On the basis of palatability scores, it appeared from the results in Table VI that temperature was without effect, but both moisture and time appeared to have significant effects. However the apparent effect of moisture was due to difference in initial quality. It was concluded that a low moisture content did not extend the storage life of dehydrated pork.

It should be noted here that the samples used in all experiments up to this point were either quickly and carefully packed on removal from the drier, or

TABLE VI

MEAN PALATABILITY SCORES AND ANALYSIS OF VARIANCE FOR DEHYDRATED PORK WITH DIFFERENT MOISTURE CONTENTS STORED IN TIN-PLATE CONTAINERS FOR ONE YEAR AT 36.7° C. (98° F.) AND 23.9° C. (75° F.)

A. Table of means

Variable under study	Mean palatability score
Moisture content (%)	
4.5	7.9
5.0	7.0
5.5	7.5
6.0	7.5
6.5	7.1
10.0	7.5
15.0	7.4
Necessary difference, 5% level	0.37
Storage temperature	
36.7° C. (98° F.)	7.4
23.9° C. (75° F.)	7.4
Storage time (months)	
Initial	7.1
1	7.6
2	7.6
3	7.2
4	7.6
6	7.7
9	7.1
12	6.9
Necessary difference, 5% level	0.37

B. Analysis of variance

Variance attributable to:	D.f.	Mean square
Moisture content	6	0.91**
Temperature	1	0
Time	6	1.26**
Residual (error)	78	0.12

** *Exceeds 1% level of statistical significance.*

held in sealed containers at 4.4° C. (40° F.) until packed. A number of samples with different moisture contents were kept on the laboratory shelves in quart sealers. These were opened occasionally for demonstration purposes. Mould growth sufficient to render the dried product inedible appeared within one month on those samples with moisture contents greater than 13%, in two months the sample with 13% moisture was also mouldy, while for moisture contents of 10% or less no moulds appeared after nine months. This indicated that moisture contents of less than 10% were desirable.

Effect of Packaging Materials

Comparison of the palatability scores in Tables I and III indicated that tin-plate was preferable to paper-bodied containers. Direct comparison of methods of packaging was therefore made on tunnel–tray-dried uncured ham with a fat content of 32% and a moisture content of 4.0%. The material was stored at 26.7° C. (80° F.), at 16 and 85% relative humidity, and at 37.8° C. (100° F.) and 11% relative humidity in tin-plate (uncompressed) and as compressed blocks in three types of substitute containers: 300 *M.S.T.* cellophane in cardboard; 300 *M.S.T.* cellophane in cardboard, finished container wax dipped (Dewey and Almy *P*-16); 300 *M.S.T.* cellophane in cardboard with an overwrap of Reynolds' metal *A*-10. The finished package contained about 300 gm. of meat and measured $1\frac{3}{4}$ in. × $3\frac{1}{2}$ in. × 4 in.

Measurements of moisture gain were made for a four-week period on packages stored at the high humidity. Comparison was made of packages carefully handled and of packages dropped six times from a height of three feet. (The latter packages were used for the quality tests since it was considered that the treatment that they had received would approximate handling conditions during transport.) Table VII shows the mean values for three packages of each type measured at weekly intervals, omitting the gains during the first week since these are complicated by increases resulting from sorption of moisture on the packaging materials. These values indicated

TABLE VII

MEAN WEEKLY MOISTURE GAIN (GM.) OF DEHYDRATED PORK IN PACKAGES WITH VARIOUS MOISTURE BARRIERS STORED AT 26.7° C. (80° F.) AND 85% RELATIVE HUMIDITY

Moisture barrier	Method of handling	
	With care	After dropping
Tin-plate	0	0
Reynolds' Metal *A*-10	0.19	0.25
Dewey and Almy *P*-16	0.21	1.19
300 *MST* cellophane	1.56	2.27

that Reynolds' metal and wax-dipping were about equally effective if carefully handled, while Reynolds' metal was the better barrier after rough treatment. The Reynolds' metal used here behaved differently from that used in another experiment (4), in that it showed no evidence of breaking down under the high humidity conditions.

The results of palatability and fluorescence measurements are shown in Table VIII; peroxide oxygen changes were again of little value (Fig. 4) although it might be noted here that the initial value was zero. The fluorescence changes indicated that storage at low temperature and low humidity was preferable while the palatability scores showed significant deterioration

TABLE VIII

MEAN PALATABILITY SCORES AND FLUORESCENCE VALUES FOR DEHYDRATED PORK STORED IN CONTAINERS WITH DIFFERENT MOISTURE BARRIERS FOR ONE YEAR UNDER VARIOUS CONDITIONS

A. Table of means

Variable under study	Mean palatability score	Mean fluorescence value
Moisture barriers		
Tin-plate	7.7	48.0
Reynolds' metal *A*-10	7.1	47.4
Dewey and Almy *P*-16	6.4	54.0
300 *MST* cellophane	6.1	50.2
Storage condition		
37.8° C. (100° F.) 11% R.H.	7.0	60.2
26.7° C. (80° F.) 85% R.H.	6.1	49.8
26.7° C. (80° F.) 16% R.H.	7.4	39.6
Necessary difference, 5% level	—	11.2
Storage time		
Initial	8.5	20.2
3	6.8	47.9
6	7.8	48.5
12	5.8	53.3
Necessary difference, 5% level	1.6	—

B. Analysis of variance

Variance attributable to:	Palatability scores		Fluorescence values	
	D.f.	Mean square	D.f.	Mean square
Moisture barriers	3	4.61	3	81
Storage condition	2	5.51	2	1275**
Storage time	2	12.32*	2	104
Residual (error)	28	2.33	26	90

* *Exceeds 5% level of significance.*
** *Exceeds 1% level of significance.*

after one year of storage. Neither criterion showed significant differences in package types. This failure to show a difference could perhaps be attributed to the fact that the mouldy portions of the samples were removed prior to testing.

Mould growth on samples stored at 26.7° C. (80° F.) and 85% relative humidity showed the relative effectiveness of the packaging materials. Samples in tin-plate did not become mouldy, in Reynolds' metal *A*-10 they were slightly mouldy after one year's storage, and in the wax-dipped cartons they were quite mouldy; samples in cellophane only were slightly mouldy after six months'

storage and inedible at the final sampling. Since samples of high moisture content when handled carefully showed no evidence of mould growth, it is likely that these samples were infected during compression.

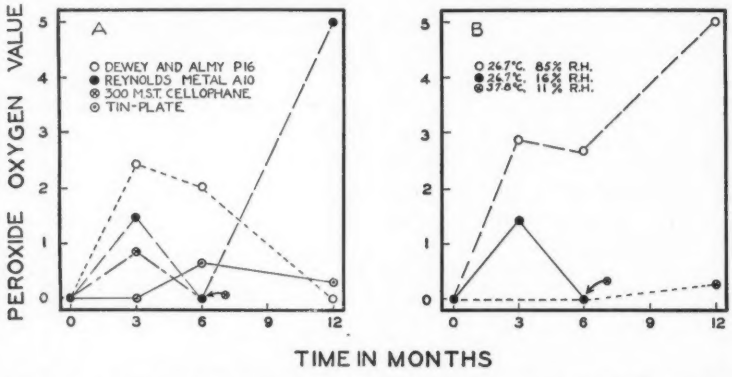

FIG. 4. *Effect of method of packaging (average for all temperatures) and of temperature (average for all methods of packaging) on the peroxide oxygen behaviour of dehydrated pork fat.*

Discussion

Throughout the study it was noted that there was generally an increase in palatability of dehydrated pork after it had been stored for a short period. This increase in palatability coincided with an increase in strong odour evident when the containers were opened: the odour disappeared after a few minutes' standing. Therefore, this initial increase in palatability was believed to result from the release during storage of volatile breakdown products formed during the drying process.

The fat fraction of fresh pork in chill or freezer storage is a relatively unstable material and the development of peroxide oxygen is an excellent indication of organoleptically detectable rancidity (1). The dried product differs markedly in behaviour; the fatty fraction of the material may show very high peroxide oxygen values without any rancidity detectable by taste panels. Explanation of this behaviour is difficult. It might be suggested that the formation of peroxide in fresh pork fat occurs at about the same rate as the deterioration actually causing rancidity in the fat, but that this deterioration is prevented as a result of the destruction of enzymes, or the formation of inhibitors during the drying process. During the storage of dried pork only the oxidative change would be taking place, hence, high peroxide oxygen values without organoleptic rancidity.

Calculations on the basis of moisture gain indicate that the product packed in Reynolds' metal should have attained a moisture content of about 8% only after one year's storage. Since moulds grew only on samples with more than 13% moisture, the presence of mould growth on the final sample must then be the result of uneven distribution of moisture through the compressed

block. The outer surfaces, where mould growth occurred, would exceed the desirable moisture limit. This feature must be considered when writing specifications for dehydrated pork to be packed in substitute containers, and to be stored for long periods at high humidity.

Acknowledgments

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RATION BISCUITS

I. FACTORS AFFECTING THE KEEPING QUALITY OF BISCUITS CONTAINING PROTEIN SUPPLEMENTS[1]

By Jesse A. Pearce[2] and J. B. Marshall[3]

Abstract

The storage life of biscuits was extended by maintaining a moisture content of 6% or less. From considerations of initial quality and of increased storage life sodium bicarbonate was superior to ammonium bicarbonate as a leavening agent, particularly at lower levels. By the same criteria, wheat germ appeared to be a more satisfactory source of protein than soy flour, dried skim milk, or dried egg yolk.

Moisture-resistant packaging materials were found necessary to prevent mould growth and maintain edibility in biscuits stored at high humidities; however, biscuits so packed deteriorated at a faster rate at high temperatures. Tin-plate containers appeared to be the most effective for long term storage at high humidities, although Reynolds' metal *A*-10 with or without an inner liner was satisfactory for storage periods of about half a year.

While none of the quality measurements used were completely reliable measures of biscuit quality, a fluorescence measurement assessed the effects of the various treatments in a relatively satisfactory manner.

Introduction

Biscuits were among the earliest forms of dehydrated food. They have been widely used for victualing ships, and as reserve rations for troops and expeditions that may be separated from ordinary sources of supply for protracted periods. The old style ships' biscuit and hard tack were made from flour, salt, soda, and a very small amount of shortening. They were very hard, dry, and difficult to masticate.

Various types of biscuits have been used extensively in normal diets. Among these are zwei-back, soda crackers, and various sorts of 'health biscuit'. Improvements in the eating quality and nutritional properties of these materials have been made by increasing the fat and protein content of the formula and by adding sugar and other ingredients to enhance the flavour. However these additions have complicated the factors affecting the keeping qualities of the product and raised questions that can be answered only by comprehensive storage experiments.

The keeping quality of foodstuffs is affected by many factors. The type of package is obviously very important. Moisture content has been shown to be an important factor in keeping quality of dried whole egg powder (14) and wheat germ (3). Sodium bicarbonate was found to affect the keeping quality

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of dried whole egg powder (7). It has also been pointed out that the use of ammonium bicarbonate in place of sodium bicarbonate as a leavening agent resulted in greater conservation of thiamin during baking (2). Various proteinagenous materials such as milk powder, wheat germ, soy flour, and dried egg yolk exhibit different keeping qualities, which in turn might be expected to influence the quality of the biscuits.

The overall change taking place during the spoilage of food material may be due to deterioration of one or more of the component fractions, i.e., fat, protein, or carbohydrate, and may be assessed by organoleptic tests without differentiating between the possible causes of the offensive taste or odour. Thus the term 'rancid' is frequently used loosely to describe taste reaction to any off-flavour although it should be reserved to describe the condition of spoiled fat. Subjecting tasters repeatedly to large numbers of samples is disturbing and generally results in a less careful judgment towards the end of a long study. A test designed to follow changes in fats, e.g. peroxide oxygen measurements, may fail completely when applied to a composite material in which spoilage of the other components may precede deterioration of the fat (11). Therefore some other objective tests have been used to assess deterioration in this foodstuff and attempts to relate these to organoleptic data have met with varying success.

Many of the factors relating to storage of foodstuffs discussed above are being investigated in these laboratories. This paper deals with an investigation of the effects of temperature, relative humidity, package materials, amount and kind of leavening agent, and type of protein, on the stability of biscuits during storage.

Materials

The standard mix for biscuits used in this experiment was: soft wheat flour, 90 lb.; whole wheat flour, 10 lb.; shortening, 12 lb.; sugar, 5 lb.; dry, powdered, skim milk, 8 lb.; salt, 1½ lb.; and sodium bicarbonate, 12 oz. The moisture content of the finished product was not to exceed 6%. All quantities and variations discussed will be in terms of this recipe.

To obtain an estimation of the effect of method of packaging, biscuits of standard mix were stored at temperatures of 26.7° (16% and 85% relative humidities), 43.3°, and 60.0° C. (80°, 110°, and 140° F.) in 300 *MST* cellophane, cellophane in tin-plate containers, tin-plate without cellophane, in Reynolds' metal *A*-10, and in Reynolds' metal plus cellophane.

For the first experiment with varying moisture content, moisture levels were adjusted, on biscuits prepared from standard mix, to 5.9, 7.1, and 10.3%, by the method described for dried whole egg powder (14). These biscuits were packaged in Reynolds' metal *A*-10 and stored at 26.7° and 43.3° C. (80° and 100° F.).

In the second experiment biscuits made from a mix using 4 oz. sodium bicarbonate and from a mix using 4 oz. ammonium carbonate were dried *in vacuo* over calcium chloride and stored at 26.7° and 43.3° C. (80° and 110° F.)

in tin-plate containers with sufficient water to bring the moisture contents to average values of 3.1%, 4.8%, 6.3%, 7.8%, and 9.1%. These levels were attained during the first week of storage.

To investigate leavening agents the standard mix was varied to include the following levels of sodium bicarbonate; 4 oz., 10 oz., 16 oz., and 22 oz.; and also 4 oz. ammonium bicarbonate and a mixture of 2 oz. ammonium bicarbonate and 2 oz. sodium bicarbonate. The finished biscuits were stored at 43.3° C. (110° F.) in Reynolds' metal *A*-10.

TABLE I

COMPOSITION OF RATION BISCUITS

Type of biscuit	Composition, %			
	Protein	Starch	Fat	Moisture
Dried milk	9.3	60.0	9.3	7.6
Wheat germ	9.3	61.6	10.6	5.4
Dried egg yolk	9.1	61.4	10.6	4.7
Soy flour	9.5	62.2	8.9	5.6

Replacing dried skim milk powder by various protein components necessitated some variation in the standard mix. These were necessary to maintain a constant ratio of protein, starch, and fat. In the first substitution, the milk powder was replaced by 6 lb., 14 oz. of defatted wheat germ; in the second, 10 lb. of powdered egg yolk replaced the skim milk and the amount of shortening was reduced from 12 lb. to 8 lb., 6 oz. The third substitution for milk powder was 7 lb., 2 oz. of debittered full fat soya flour with a reduction of shortening from 12 lb. to 10 lb., 6 oz. The protein, starch, fat, and moisture contents of the resulting biscuits are shown in Table I. These biscuits were stored at 26.7° (16% and 85% relative humidities), 43.3°, and 60.0° C. (80°, 110°, and 140° F.) in 300 *MST* cellophane, in Reynolds' metal *A*-10, and in Reynolds' metal plus cellophane.

Methods

Since the ultimate criterion of quality must be edibility of the product, palatability measurements were made by panels of 14 people, each person sampling the biscuits and commenting as follows: excellent, very good, good, fairly good, fair, fair to poor, poor, very poor, and inedible. For ease in averaging the final data, these descriptive categories were assigned numerical values of 8 to 0, respectively.

A fluorescence measurement had proved useful as a test of quality in dried whole egg powders (6, 10). It appeared to measure breakdown of the proteins present in dried egg powders (4), and fluorescence changes have been observed in foods of high carbohydrate content (5). In addition, all the protein com-

ponents selected for use in this study were known to have a fluorescence that varied more or less with storage conditions (3, 4, 5); therefore, this measurement seemed appropriate for the present study. Fluorescence measurements were made in a manner similar to the revised method used for dried whole egg powder (6); the results are recorded in units of the Coleman photofluorometer.

Changes in acidity of stored flour and wheat germ are known to occur (1, 9). Work on dried egg powders indicated that the pH of potassium chloride extracts of defatted powder was related to storage treatment (14). Since it has been noted that alcohol extracts contained a portion of the fluorescing substances, the amount varying in quantity with the quality of the egg powder (4), it was believed that pH measurements on alcohol extracts of biscuits might prove to be a good measure of quality. Therefore pH measurements were made on alcohol (95%) extracts of the whole biscuits after dilution of the alcohol extract to 50%, on extracts of defatted biscuits in 10% sodium chloride, and on extracts of whole biscuits in 10% potassium chloride. This last type of extract should reflect changes in acidity occurring in the whole biscuit, the second, changes in the non-fatty portion, and the first, changes in the fatty portion although some amino acids and protein matter might be present (8). The average pH of the various solvents was as follows: 10% potassium chloride, 5.2; 10% sodium chloride, 5.5; and alcohol, when diluted, 6.2.

To reduce interference resulting from deterioration of the fat, the most stable shortening obtainable was used (13). Nevertheless, it was felt desirable to measure peroxide oxygen development in the fat fraction of the biscuits. These measurements were made on fat extracted from the biscuits; the technique of this determination has been described (12). Development of peroxide oxygen values was slow, a month's storage at 60.0° C. (140° F.) and eight months' storage at 26.7° C. (80° F.) being required to bring the value to about 1 ml. 0.002 *N* thiosulphate per gram of extracted fat. The biscuits had deteriorated to a marked extent for other reasons and hence peroxide oxygen values were of little importance and are not discussed.

The withdrawal of packages at the various time periods was done by a randomized procedure. Sampling the contents of the selected packages for palatability scores and objective tests differed somewhat. Four biscuits from a package were broken into four pieces each and one piece sampled by a taster, while for the objective measurements one analysis was done on each of two biscuits from the same package. This method was used because the error of the quality measurements, appreciable between biscuits, was thought to be greater than between packages. Furthermore, it was known that the analytical error was much smaller than the between-biscuit error.

Results

Effect of Packaging

The data obtained with five different types of packages are shown in Fig. 1. At 60·0° C. (140° F.) fluorescence development in biscuits stored for four weeks differed for each type of package. The material packaged in cellophane

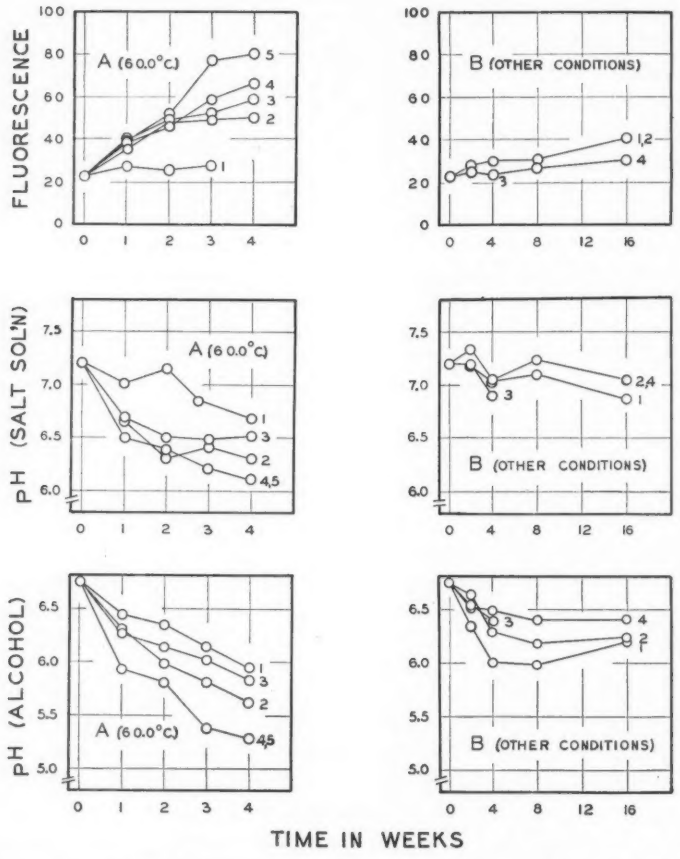

FIG. 1. *Changes in fluorescence values and pH of extracts of biscuits stored in different packaging materials.*

A, at 60.0° C. (140° F.): Curve 1, 300 MST cellophane, Package (a); Curve 2, in Reynolds' metal A-10, Package (b); Curve 3, in 300 MST cellophane and Reynolds' metal A-10, Package (c); Curve 4, in tin-plate, Package (d); Curve 5, in 300 MST cellophane and tin-plate, Package (e).

B, at other conditions: Curve 1, Packages (b), (c), (d), and (e), at 43.3° C. (110° F.); Curve 2, Package (a) at 26.7° C. (80° F.) and 85% relative humidity; Curve 4, all other packages at 26.7° C. (80° F.).

alone developed fluorescing substances more slowly than any of the others. Similar changes occurred in biscuits packed in tin-plate and in Reynolds' metal, except that slightly higher values developed in the biscuits packaged in the former: the additional cellophane wrapping seemed to accentuate changes occurring in biscuits stored in Reynolds' metal or in tin-plate.

Fluorescence values showed no difference in biscuits packaged in various materials and stored for 16 wk. at 43.3° C. (110° F.) and at 26.7° C. (80° F.)

and 16% relative humidity although fluorescence development was slightly greater at the higher temperature. Biscuits packaged in cellophane and stored for four weeks at 26.7° C. (80° F.) and 85% relative humidity developed fluorescence values at the same rate as biscuits in other packages, but at six weeks they showed some mould growth and at eight weeks were too mouldy for measurements. Biscuits in the other types of packages behaved in the same manner as those stored at the lower humidity.

Increases in acidity were noted in biscuits stored at all conditions, the greatest changes occurring in those packaged in tin-plate and stored at 60° C. (140° F.). The least change occurred in the material wrapped in cellophane only. At 43.3° C. (110° F.) cellophane wrapped biscuits developed acid products more slowly than biscuits packed in other materials. Biscuits packaged in cellophane and stored at 26.7° C. (80° F.) and 85% relative humidity decreased in pH more rapidly than biscuits in all other types of package stored at both humidities. Changes in pH of the alcohol extracts of these biscuits showed the same trends as that of the sodium chloride extracts, although the general level was lower.

On the basis of the foregoing measurements there seemed to be little difference in the behaviour of materials packaged in Reynolds' metal and tin-plate, except at high temperatures, where it appeared that tin-plate had a more drastic effect on the contents than the substitute packaging materials. Most surprising was the adverse effect resulting from the addition of a cellophane wrap on materials stored at the high temperature, indicating that under this condition materials with higher moisture vapour transmission extended storage life. As a result of this experiment, cellophane packages seemed to merit further study. Reynolds' metal was selected also for use in further studies, since any changes occurring in tin-plate could be expected to be even more drastic at high temperatures and similar at low temperatures.

Effect of Moisture

Table II, showing mean values, indicates the effect of moisture levels on biscuits of standard mix as assessed by fluorescence values and pH measurements on the sodium chloride extract of the defatted biscuits. These results show the effect of moisture content in increasing the development of fluorescing substances. An increase in moisture content from 6 to 7% caused a significant increase in fluorescing substances. A further increase to 10% had a less marked effect on the rate of fluorescence development. Since the range of conditions was limited, pH measurements did not show a significant effect due to moisture content although the trends indicated by the mean values support observations on the basis of fluorescence development.

It was concluded from this experiment that an investigation of a still greater range of moisture levels was necessary. It was believed expedient to include a study of the effect of different leavening agents. The results of this second experiment are given in Tables III and IV.

TABLE II

EFFECT OF MOISTURE ON KEEPING QUALITY OF BISCUITS MADE FROM THE STANDARD MIX AND STORED 15 WK. AT 43.3° C. (110° F.)

A. Table of means

Variable under study		Fluorescence (photofluorometer units)	pH of sodium chloride extract
Time, wk.	Initial	27.1	7.92
	1	27.4	7.44
	2	29.7	7.09
	6	32.5	7.16
	15	33.1	6.85
Necessary difference, 5% level		1.68	0.20
Moisture content, %	5.9	27.5	7.38
	7.1	30.4	7.25
	10.3	32.0	7.25
Necessary difference, 5% level		1.30	—
Temperature	26.7° C. (80° F.)	28.1	7.34
	43.3° C. (110° F.)	59.9	7.25

B. Analysis of variance

Variance attributable to:	D.f.	Mean square	
		Fluorescence (photofluorometer units)	pH of sodium chloride extract
Temperature	1	102.67**	0.0599
Moisture	2	51.26**	0.0517
Time	4	46.48**	1.0228**
Temperature × moisture	2	10.31*	0.0909
Time × temperature	4	40.60**	0.0295
Moisture × time	8	6.32*	0.0332
Temperature × moisture × time	8	1.60	0.0225

* *Exceeds 5% level of statistical significance.*
** *Exceeds 1% level of statistical significance.*

TABLE III

PALATABILITY SCORES OF STORED BISCUITS AT DIFFERENT MOISTURE LEVELS, AVERAGE FOR TWO TYPES OF LEAVENING AGENT

Moisture content, %	Palatability scores	
	43.3° C. (110° F.) 11 wk.	26.7° C. (80° F.) 15 wk.
3.1	3.7	5.4
4.8	3.9	5.4
6.3	3.6	5.2
7.8	2.7	4.4
9.1	2.8	4.2
Necessary difference, 5% level	0.7	0.7

TABLE IV

EFFECT OF MOISTURE ON KEEPING QUALITY OF BISCUITS CONTAINING DIFFERENT LEAVENING AGENTS AND STORED 11 WK. AT 43.3° C. (110° F.)

A. Table of means

Variable under study	Fluorescence (photofluorometer units)		pH of potassium chloride extract	
	Biscuit 1	Biscuit 2	Biscuit 1	Biscuit 2
Time, wk. Initial	19.2	28.7	6.60	6.60
1	20.6	30.6	6.60	6.69
3	19.3	31.4	6.58	6.68
7	16.4	24.9	6.47	6.50
11	21.1	34.6	6.47	6.48
Necessary difference, 5% level	2.9	4.7	0.04	0.02
Moisture content, %				
3.1	17.9	36.8	6.55	6.63
4.8	17.7	27.2	6.56	6.64
6.3	18.7	32.8	6.52	6.62
7.8	19.8	38.2	6.51	6.57
9.1	22.7	38.2	6.43	6.47
Necessary difference, 5% level	3.4	5.3	0.05	0.03

B. Analysis of variance

Variance attributable to:	D.f.	Mean square			
		Fluorescence (photofluorometer units)		pH of potassium chloride extract	
		Biscuit 1	Biscuit 2	Biscuit 1	Biscuit 2
Time	3	44.01**	164.85**	0.0845**	0.1245**
Moisture content	4	33.35**	106.45**	0.0186**	0.0409**
Moisture content × time	12	7.99	24.98	0.0048**	0.0837**
Between biscuits	20	4.74	12.80	0.0011	0.0003

****Exceeds 1% level of statistical significance.***

Table III shows the mean palatability scores (mean scores by eight tasters) combined for both types of biscuits (4 oz. sodium bicarbonate compared with 4 oz. ammonium bicarbonate as leavening agent) after storage. Statistical analyses showed that the types of biscuits did not differ, the only significant effects being those due to tasters, to moisture contents, and temperature. It is evident from this table that the biscuits between 3.1 and 6.3% moisture did not differ after storage but were of higher quality than biscuits at 7.8 and 9.1% moisture.

The quality measurements used, i.e. fluorescence value, pH of potassium chloride, sodium chloride, and alcohol extracts were generally not satisfactory.

The only significant changes were in fluorescence substances and in pH of the potassium chloride extracts of biscuits stored at 43.3° C. (110° F.). Only the significant results are given in Table IV; these show considerable variation in the values obtained by the chemical analyses, but tend to support the conclusions drawn from palatability scores.

The above results indicate that the storage life of biscuits was extended by maintaining moisture content in the biscuits of 6% or less.

Effect of Soda Level

The results of objective tests on biscuits containing varying amounts of sodium bicarbonate, ammonium bicarbonate, and both sodium and ammonium bicarbonate are shown in Fig. 2.

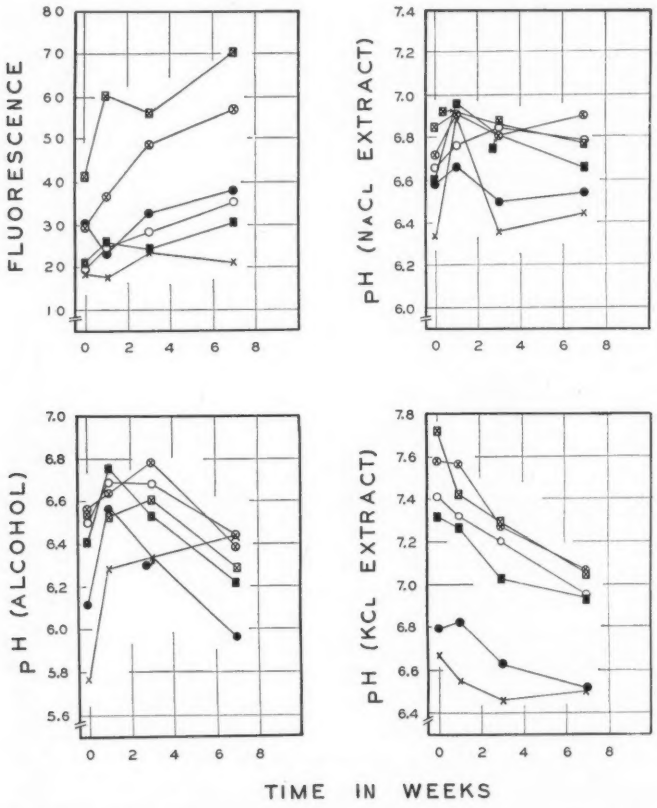

FIG. 2. *Changes in quality measurements of biscuits containing various amounts of leavening agent when stored at 43.3° C. (110° F.).* × = *4 oz. sodium bicarbonate;* ○ = *10 oz. sodium bicarbonate;* ⊗ = *16 oz. sodium bicarbonate;* ⊠ = *22 oz. sodium bicarbonate;* ● = *4 oz. ammonium bicarbonate;* ■ = *4 oz. of each of sodium bicarbonate and ammonium bicarbonate.*

No conclusions could be drawn from pH measurements on the alcohol extracts and on extracts of the defatted biscuits in 10% sodium chloride solution. The trends here were quite different from those observed in the experiment with different packages. An initial rise in pH occurred that is at present unexplainable.

The pH measurements on 10% potassium chloride extracts of the whole biscuits followed trends similar to those previously observed. The rate of decrease in pH was greater for biscuits containing 16 oz. and 22 oz. sodium bicarbonate. Biscuits containing 10 oz. sodium bicarbonate and both sodium and ammonium bicarbonate behaved similarly and changed more slowly than the biscuits containing the higher concentrations of leavening agent. The biscuits containing 4 oz. of leavening agent changed slowly; those with 4 oz. sodium bicarbonate changing least of all.

Fluorescence measurements indicated greater changes at the higher soda levels, with biscuits containing ammonium bicarbonate behaving similarly to biscuits containing 10 oz. sodium bicarbonate. There was a more rapid increase in fluorescing substances in biscuits containing both leavening agents than in biscuits containing the smallest quantity of sodium bicarbonate. Both of these quality tests indicated that the lower the soda level the more desirable was the biscuit, both in initial quality and in maintenance of quality during storage.

The mean palatability scores obtained on the biscuits initially and after seven weeks' storage are given in Table V. Increasing soda level evidently resulted in a definite decrease in initial flavour score. Seven weeks' storage lowered the flavour ratings, with the exception of the biscuit containing ammonium carbonate, which did not change in score during this storage period. However, the use of ammonium carbonate either alone or in combination with sodium carbonate resulted in reduced initial palatability scores. The biscuit containing 22 oz. of sodium bicarbonate deteriorated significantly more than the other types of biscuit. These features were assessed statistically on duplicate trials by the taste panel.

TABLE V

MEAN PALATABILITY SCORES OF BISCUITS CONTAINING VARIOUS AMOUNTS OF LEAVENING AGENT, STORAGE AT 43.3° C. (110° F.)

Leavening agent		Palatability scores	
		Initial	At seven weeks
Sodium bicarbonate,	4 oz.	8.1	7.5
	10 oz.	7.7	7.1
	16 oz.	7.2	6.4
	22 oz.	6.8	5.2
Ammonium bicarbonate	4 oz.	6.3	6.4
Sodium bicarbonate 4 oz. / Ammonium bicarbonate 4 oz.		7.3	6.8

From the above results, it is evident that sodium bicarbonate was a more desirable leavening agent, and that smaller quantities were most desirable.

Effect of Protein Components

The mean values and an analysis of variance of fluorescence measurements made on biscuits with different protein components are shown in Table VI. Measurements of pH were omitted because they were of little value in interpreting quality differences. The behaviour of the biscuits at 60.0° C. (140° F.) was somewhat different from that at the lower temperatures. The results at this high temperature show the wheat germ and soya flour biscuits to have generally lower fluorescence value, while at the lower temperatures only the

TABLE VI

FLUORESCENCE CHANGES IN BISCUITS WITH DIFFERENT PROTEIN COMPONENTS

A. Table of means

Variable under study	Fluorescence (photofluorometer units)				
	60.0° C. (140° F.)	43.3° C. (110° F.)	26.7° C. (80° F.) (16% *R.H.*)[3]	26.7° C. (80° F.) (85% *R.H.*)[3]	
Type of biscuit					
Skim milk powder	37.29	29.92	24.96	24.10[1]	29.03[2]
Wheat germ	32.35	27.90	27.13	26.30	26.94
Powdered egg yolk	43.05	40.45	40.31	36.97	38.40
Soya flour	31.16	29.60	28.53	31.03	33.03
Necessary difference	3.46	3.13	2.10		
Type of package					
300 *MST* cellophane	32.74	31.07	30.90	29.53	
Reynolds' metal *A*-10	36.77	32.85	30.56		31.65
Reynolds' metal and cellophane	38.38	31.98	29.23		27.64
Necessary difference	3.00	—	—		
Storage time					
Initial	26.28	26.28	26.28	26.28	26.28
1 day	30.26	30.89	—	—	—
3 days	31.65	30.60	—	—	—
7 days	35.17	28.14	29.24	27.44	28.50
15 days	40.72	—	—	—	—
21 days	—	30.70	28.25	29.29	31.59
31 days	42.02	—	—	—	—
49 days	—	30.22	29.18	32.08	30.69
105 days	—	34.55	30.51	—	29.15
217 days	—	38.66	33.98	—	36.42
Necessary difference	3.87	4.14	2.35		

[1] *Values in this column for biscuits packaged in 300 MST cellophane.*

[2] *Values in this column combined results for biscuits packaged in Reynolds' metal A-10 and in Reynolds' metal and cellophane.*

[3] *R.H. = relative humidity.*

TABLE VI—*Concluded*

FLUORESCENCE CHANGES IN BISCUITS WITH DIFFERENT PROTEIN COMPONENTS—*Concluded*

B. Analysis of variance

Variance attributable to:	60.0° C. (140° F.)		43.3° C. (110° F.)		26.7° C. (80° F.) (16% *R.H*)[3]	
	D.f.	Mean square	D.f.	Mean square	D.f.	Mean square
Package	2	336.81**	2	44.20	2	31.12
Biscuits	3	880.71**	3	1375.88**	3	1419.91**
Time	4	666.90**	6	295.33**	4	120.99**
Biscuits × package wrappers	6	84.14	6	183.92**	6	16.88
Package × times	8	65.73	12	22.32	8	25.02
Biscuits × times	12	121.29*	18	48.17	12	9.46
Biscuits × package × times	24	42.14	36	50.10	24	15.50
Between biscuits	60	23.81	84	26.25	60	17.36

* *Exceeds 5% level of statistical significance.*
** *Exceeds 1% level of statistical significance.*

egg powder biscuit had high values. These measurements show cellophane, when used alone, to be a preferable wrap at the highest temperature, although it was definitely undesirable at the lowest temperature if the humidity was high. This undesirability was due to mould growth resulting from increased moisture contents of the biscuits rather than to changes detected by fluorescence measurements. No difference in packages was observed at 43.3° C. and 26.7° C. (110° and 80° F.) at low humidity. Fluorescing substances generally increased with time for all types of biscuits; although wheat germ biscuits reached a maximum value at 15 days' storage.

Initial palatability scores of biscuits with various protein components were: skim milk powder, 5.2; soy flour, 5.2; dried egg yolk, 5.5; and wheat germ, 6.0. Statistical analysis of the data indicated that the wheat germ biscuit was significantly better than the others.

Statistical analysis of the palatability scores on stored biscuits with various protein components (Table VII) lead to conclusions slightly different from those cited above. Wheat germ and soya biscuits were again most stable at 60.0° C. (140° F.) while the wheat germ biscuit was the most stable at 43.3° C. (110° F.) and 26.7° C. (80° F.) (16% relative humidity). Egg yolk biscuits and milk biscuits deteriorated most rapidly at the two higher temperatures. Cellophane was the most satisfactory packaging material at 60° C. (140° F.), no difference occurring at the lower temperatures unless the humidity was high. At 60.0° C. (140° F.) decrease in palatability of biscuits that were double wrapped was more rapid than for biscuits in either of the single wrapped packages. Highly moisture-vapour resistant wrappings caused more drastic reduction in palatability in biscuits containing milk powder when stored at the high temperature.

TABLE VII

PALATABILITY CHANGES IN BISCUITS WITH DIFFERENT PROTEIN COMPONENTS

A. Table of means

Variable under study	Palatability scores				
	60.0° C. (140° F.)	43.3° C. (110° F.)	26.7° C. (80° F.) (16% *R.H.*)[3]	26.7° C. (80° F.) (85% *R.H.*)[3]	
Type of biscuit					
Skim milk powder	3.4	4.6	5.5	4.1[1]	5.2[2]
Wheat germ	4.9	5.3	5.9	4.1	5.8
Powdered egg yolk	3.5	3.8	4.3	3.3	4.5
Soya flour	4.6	4.9	5.4	4.3	5.3
Necessary difference	0.26	0.21	0.17		
Type of package					
300 *MST* cellophane	4.4	4.7	5.2	3.9	
Reynolds' metal *A*-10	4.1	4.6	5.3		5.3
Reynolds' metal and cellophane	3.8	4.6	5.3		5.2
Necessary difference	0.23	—	—		
Storage time					
Initial	5.5	5.5	5.5	5.5	5.5
1 day	5.4	5.2	—	—	—
3 days	4.6	5.2	—	—	—
7 days	4.0	4.8	5.2	5.2	5.6
15 days	3.9	—	—	—	—
21 days	—	4.7	5.1	3.9	5.0
31 days	2.4	—	—	—	—
49 days	—	4.4	5.0	2.8	5.5
105 days	—	4.1	5.2	—	5.0
217 days	—	4.0	5.9	—	5.0
Necessary difference	0.29	0.27	0.19		

B. Analysis of variance

Variance attributable to:	60.0° C. (140° F.)		43.3° C. (110° F.)		26.7° C. (80° F.) (16% *R.H.*)[3]	
	D.f.	Mean square	D.f.	Mean square	D.f.	Mean square
Package	2	2.08**	2	0.10	2	0.04
Biscuits	3	8.35**	3	8.25**	3	7.49**
Time	4	14.61**	6	2.69**	4	1.48**
Biscuits × package	6	0.53**	6	0.22	6	0.16*
Package × times	8	0.38**	12	0.08	8	0.08
Biscuits × times	12	0.44**	18	0.35**	12	0.06
Biscuits × package × times	24	0.12	36	0.11	24	0.05

[1] *Values in this column for biscuits packaged in 300 MST cellophane.*

[2] *Values in this column combined results for biscuits packaged in Reynolds' metal A-10, and in Reynolds' metal and cellophane.*

[3] *R.H. = relative humidity.*

* *Exceeds 5% level of statistical significance.*

** *Exceeds 1% level of statistical significance.*

Unfortunately the skim milk powder biscuit had a moisture content of 7.6% instead of the 6% or less required. The foregoing data on moisture levels (Tables II, III, and IV) permitted some estimation of the error introduced by this difference. Calculations indicated that the difference in moisture contents of the wheat germ and skim milk powder biscuits was not great enough to account for the poorer storage life of the latter material. Therefore, it is believed that the validity of the conclusions from this portion of the investigation will not be vitiated by the higher moisture content of the skim milk powder biscuit.

From considerations of both initial palatability and keeping quality, wheat germ biscuit was the most desirable source of protein. Storage at 26.7° C. (80° F.) in a dry atmosphere induced little change and single wrappings of moisture-resistant packaging materials appeared to be the most desirable for general use.

Relation between Palatability and Objective Measures of Quality

The palatability test, on the whole, was the most satisfactory measure of quality of the biscuits, but like most organoleptic tests the error of replication was high. There was, furthermore, a noticeable tendency for the scores to increase toward the end of the experiment, as can be seen in Table VII.

To assess the merits of the various objective tests correlation coefficients were calculated. These were limited to calculations on palatability–fluorescence, and flourescence–pH relations. As mentioned previously, calculations on peroxide oxygen measurements would have contributed nothing. Correlations between the various pH measurements were so small that calculations were useless. In no instance were related data on pH measurements and palatability available.

For each type of biscuit correlations between fluorescence values and palatability scores were found to be negative, highly significant, but generally low in magnitude, e.g. skim milk powder biscuit, −.687**; wheat germ biscuit, −.315**; dried egg yolk biscuit, −.386**; and soy flour biscuit, −.528**. If the data for any one variable were averaged over all others high correlations between fluorescence and palatability were obtained, e.g. for time periods, −.897; wraps, −.956; biscuit types, −.828; and for storage conditions, −.854.

Correlations between fluorescence values and pH measurements were calculated for the skim milk powder biscuit. Again, the correlations were low; −.245*, −.082, and −.088 for fluorescence with pH's of alcohol extracts, sodium chloride extracts, and potassium chloride extracts respectively. Averaging the data for all variables at each time period showed higher correlations, −.675, −.627, and −.614 between fluorescence and the respective extracts.

* *Exceeds 5% level of statistical significance.*
** *Exceeds 1% level of statistical significance.*

While none of the quality measurements correlated closely with palatability scores, the fluorescence measurement reflected storage treatment with a fairly high degree of accuracy. On the basis of the present experiment fluorescence measurements supported by palatability evaluation of initial and final samples would probably give a satisfactory picture of the stored biscuits.

Moisture Changes During Storage in Substitute Containers

The average moisture gain in grams is given in Table VIII for biscuits packaged as described for the preliminary experiment, where an average weight of 74 gm. per package of skim milk powder biscuits was stored at 26.7° C. (80° F.) and at 85% relative humidity. The dimensions of the finished package were 2 in. × 2 in. × 2 in. It is evident that 300 *M.S.T.* cellophane is unsatisfactory under these conditions and Reynolds' metal *A*-10 permits a small moisture increase. This small moisture increase did not appear to affect the quality of the biscuits in the package (Fig. 1).

TABLE VIII

MOISTURE GAIN OF SKIM MILK POWDER BISCUITS PACKAGED IN VARIOUS MATERIALS AND STORED AT 26.7° C. (80° F.) AND 85% RELATIVE HUMIDITY

Type of package	Gain per package (gm.) after storage for:				
	2 wk.	4 wk.	6 wk.	8 wk.	16 wk.
300 *MST* cellophane	2.93	4.97	7.39[1]	—[2]	—[2]
Reynolds' metal *A*-10, with and without 300 *MST* cellophane liner	0.14	0.28	0.32	0.44	0.57
Tin-plate, with and without 300 *MST* cellophane liners	0.02	0.01	0.02	0.03	0

[1] *Some mould growth on biscuits.*

[2] *Mould growth too heavy to permit measurement.*

The second experiment included only 300 *M.S.T.* cellophane and Reynolds' metal *A*-10 packages, the latter with and without cellophane inner liners. The moisture gain for an average weight of 132 gm. of biscuit per package of dimension 2 in. × 3 in. × 3 in. is given in Table IX. One outstanding feature of these results is that the inner cellophane liner contributed additional moisture protection; this was not evident from the earlier experiment. Little information can be gathered from the breakdown of the data into biscuit types, although there is some evidence that the package containing biscuits made with powdered egg yolk picked up moisture at a slightly faster rate than the others.

It was observed during these storage trials that the random sampling technique used in selecting these packages could still be applied at the 15-wk. samplings. However, at the 31 wk. period it was no longer applicable to the packages stored at 26.7° C. (80° F.) and 85% relative humidity as a result of failure of the packaging material. Under this condition of high humidity

TABLE IX

MOISTURE GAIN OF ALL TYPES OF BISCUITS PACKAGED IN VARIOUS MATERIALS AND STORED AT 26.7° C. (80° F.) AND 85% RELATIVE HUMIDITY

Variable under investigation	Gain per package (gm.) after storage for:				
	1 wk.	3 wk.	7 wk.	15 wk.	31 wk.
By package types					
300 *MST* cellophane	4.81	8.84	14.72	—[1]	—[1]
Reynolds' metal *A*-10	0.46	0.50	0.75	1.21	2.34
Reynolds' metal with cellophane liner	0.40	0.43	0.64	1.07	2.01
By biscuit types[2]					
Skim milk powder	0.38	0.39	0.58	0.98	1.77
Wheat germ	0.41	0.46	0.66	0.83	2.56
Powdered egg yolk	0.50	0.52	0.98	1.89	2.34
Soy flour	0.42	0.48	0.54	0.87	1.85

[1] *Mould growth too heavy to permit measurement.*

[2] *Mean values for samples in Reynolds' metal A-10 with and without cellophane liner.*

some separation of layers of the Reynolds' metal wrapper had occurred with resulting loss in protection. This is supported by the data for packages containing wheat germ and soy flour biscuits: even though only apparently undamaged packages were chosen for measurements at this last sampling, the moisture gain during the last 16 wk. was higher than would have been expected from the data for the first 15 wk. At the same temperature, and 16% relative humidity many packages made from these substitute materials were destroyed by crickets, which infested the storage room during the 15 to 31 wk. interval. Again, the final measurements were made only on undamaged packages.

Changes in moisture content of the biscuits were measured during the course of the experiment on protein constituents. The only significant changes in moisture content occurred in cellophane wrapped biscuits stored at 60.0° C. (140° F.), and at 26.7° C. (80° F.) and 85% relative humidity. At the higher temperature the average moisture content for all types of biscuits was reduced from 5.8% to 2.7% in 15 days; while at 26.7° C. (80° F.) and 85% relative humidity the average moisture content for all types of biscuits increased to 12.8%.

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RESIN-RUBBER FROM CANADIAN GROWN PLANTS

III. PRELIMINARY PILOT PLANT EXTRACTION OF GUM FROM MILKWEED LEAVES[1]

By J. Klassen[2] and N. H. Grace[3]

Abstract

A pilot plant method for the extraction of resin–rubber gum from milkweed leaves involved a preliminary water extraction at the boiling point for 15 min. and a 1% alkali cook at 10 lb. steam pressure for 1½ hr. The cooked leaves were then filtered and washed to a pH of less than 11.5 and milled in a 120 gal. pebble mill for 3½ hr. The resulting slurry was passed through a 60 mesh screen and the undersize then subjected to froth flotation. The resin–rubber fraction was concentrated in the froth, which, after thickening, was fed into a pebble mill for agglomeration.

Introduction

Preliminary studies (1) indicated possible value of the resin–rubber from milkweed as a blending agent for the synthetic rubber GR-S. Possible industrial utilization of the resin–rubber fraction as such, or of the components separately, obviously is dependent on the economics of the extraction process. These considerations led to the adaptation of the laboratory extraction method (2) to the pilot plant scale. Therefore this work had two objectives: first, the adaptation of laboratory methods to the pilot plant, and second, preparation of a suitable amount of milkweed resin–rubber gum for immediate test as a blending agent. Stress has been laid on the rapid extraction of gum for test purposes. Maximum yields, minimum cost, and continuous plant operation are subsequent phases, and beyond the scope of this communication. Even when most of the difficulties of scale had been overcome the rubber would not agglomerate in the large mills and the additional process of flotation was introduced (4).

Procedure

The procedure finally employed in the pilot plant is diagrammatically represented in the flow sheet (Fig. 1). The cooking operation involved a 15-min. water extraction at the boiling point and a 1% sodium hydroxide extraction at 10 lb. steam pressure for 1½ hr. The cooked leaves were filtered hot and washed to a pH of 11.0 to 11.5 instead of to the previous pH of 9.5 (2), thereby effecting a considerable saving in time. The washed leaves were pebble-milled for 3½ hr., screened, and the undersize passed through a flotation cell. The resin–rubber fraction was concentrated in the froth (4); this concentrate, often very watery, was thickened and then agglomerated in

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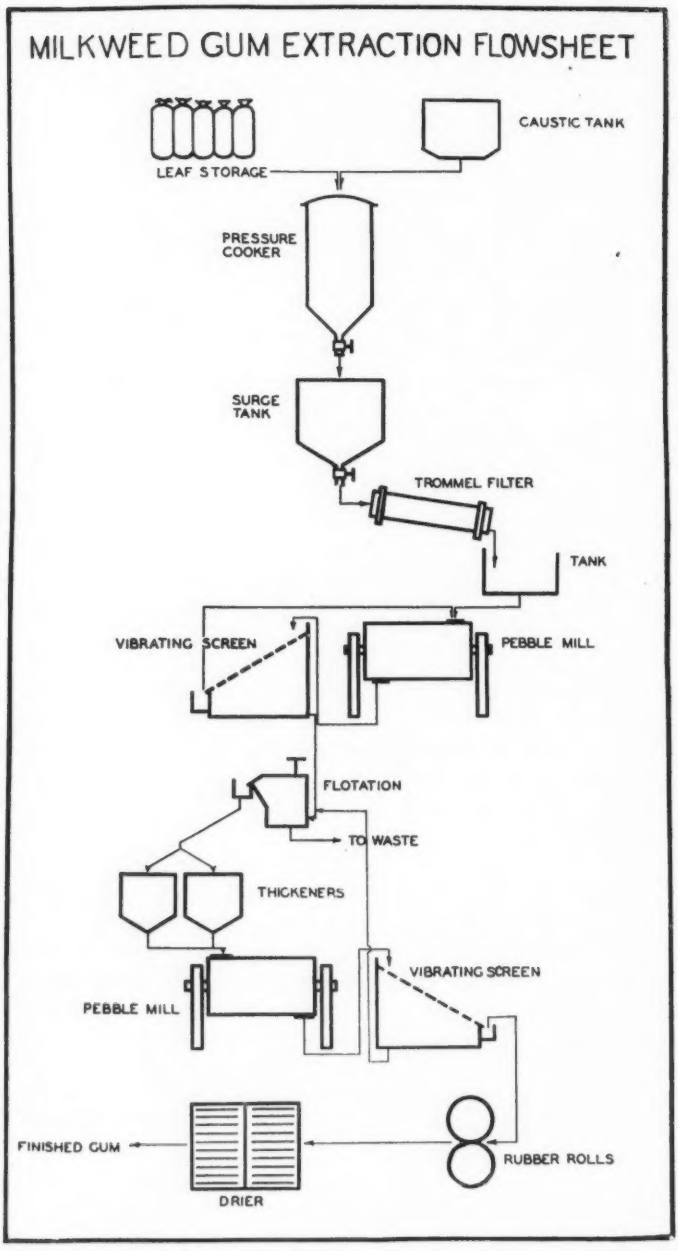

FIG. 1. *The flowsheet for the pilot plant extraction of resin–rubber gum from milkweed leaves.*

a pebble mill. After agglomeration the gum was picked out by hand for it usually consisted of one or two pieces weighing from 6 to 14 lb. depending on the grade and amount of feed. The gum was then creped on rubber washing rolls, spread on wire trays, and dried in either a vacuum or air drier. The moisture content was thus reduced from 20–30% to less than 1%, and the gum was ready for test.

The procedure may be divided into a number of unit processes, namely, cooking, filtering, washing, milling, screening, flotation, thickening, agglomeration, and drying. Though all these steps are independent, a change in any one may affect some or all of the succeeding steps. In assessing the various unit processes, the results shown are the yield of gum obtained as agglomerated in a bank of seven 1-gal. laboratory pebble mills. The description and discussion of the pilot plant extraction procedure will be undertaken as a series of unit processes in the order illustrated in the flow sheet.

Pilot Plant Unit Processes

Cooking

The pilot plant has one open and two pressure cookers (Fig. 3). The open cooker, 32 in. diam. by 48 in. high with a dished bottom fitted with a 6-in. gate valve, is heated by direct injection of steam. The two pressure cookers are 32 in. diam. by 64 in. high with a 45° conical bottom fitted with a 6-in. gate valve. Both cookers have a maximum operating pressure of 50 lb. and are heated by the direct injection of steam, or a mixture of steam and air to reduce implosions.

Eighty to ninety pounds of leaves were loaded into a pressure cooker filled three-quarters full with water and were extracted at the boiling point for 15 min. The water dissolved about 35% of the dry weight of the leaves. This extract was drained through a screen placed over the gate value, the valve was then closed, and the screen removed. The cooker was then filled three-quarters full with a 1% sodium hydroxide solution and steam injected until a pressure of 10 lb. was reached. The cook was blown into the surge tank after $1\frac{1}{2}$ hr.

Preliminary water extraction, volume of solution, degree of alkalinity, steam pressure, and duration of cooking were factors believed worthy of consideration. Each of these has been studied with the exception of volume of solution.

Increased yield resulting from a preliminary water extraction may be seen from the data in Fig. 2 and Table I.

Effects of alkali concentration are also shown in Fig. 2. With other operating conditions at their optimum, gum recovery was greater at 1% alkali than at lower concentrations. In general (Table I) concentrations of alkali greater than 1% did not result in practical increases in yield. Above 3% sodium hydroxide a sharp decline in gum yield occurred.

The effects of pressure and duration of the cook are indicated in Table I. A comparison of the water-extracted leaves cooked at 10 and 40 lb., respec-

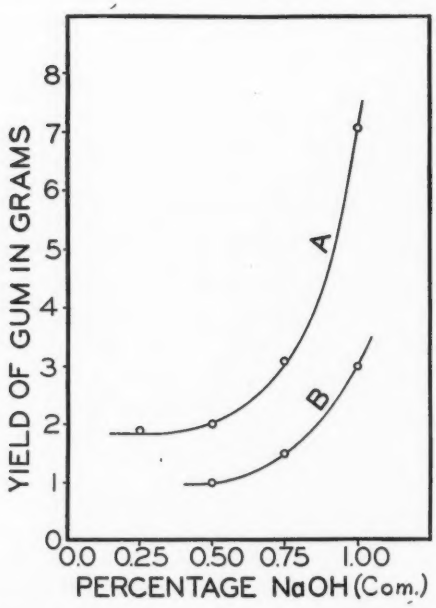

FIG. 2. *Effects of sodium hydroxide concentration on the yield of gum from 200 gm. of dry leaves. A. Preliminary water extraction. B. No water extraction.*

tively, shows that the yield of gum was substantially greater with the lower pressure cook. A two hour cook was satisfactory.

Filtering and Washing

While filtering and washing may be considered as separate unit processes, in plant operations, they are so closely interwoven that separate discussion is inadvisable. Two types of filters were studied during this investigation. The first type was a 4 by 4 by 4 ft. box filter. The other type studied was a trommel filter. This is a rotating (32 r.p.m.), cheesecloth covered cylinder, open at both ends, 2 ft. in diam. by 8 ft. long, with a water spray in the central third portion.

The cooked milkweed leaves, ranging from particles 4 in. long to minus 100 mesh, presented filtering difficulties, causing serious binding of filtering media. A box filter fitted with mosquito wire screening operating under a hydrostatic head of 3 ft. filtered, after two hours' operation, at the rate of $\frac{1}{2}$ in. per hr. Agitation increases the pre-binding period but the rate of filtration is still too slow. Four layers of cheesecloth over the wire screen improved filtration, but bound just as firmly. Plant debris lodged over the pores and holes and was held in place by hydrostatic pressure.

The introduction of a trommel filter decreased binding. As the cooked leaves passed through this piece of equipment, they were filtered, washed, and finally filtered to yield a product closely resembling cooked, drained

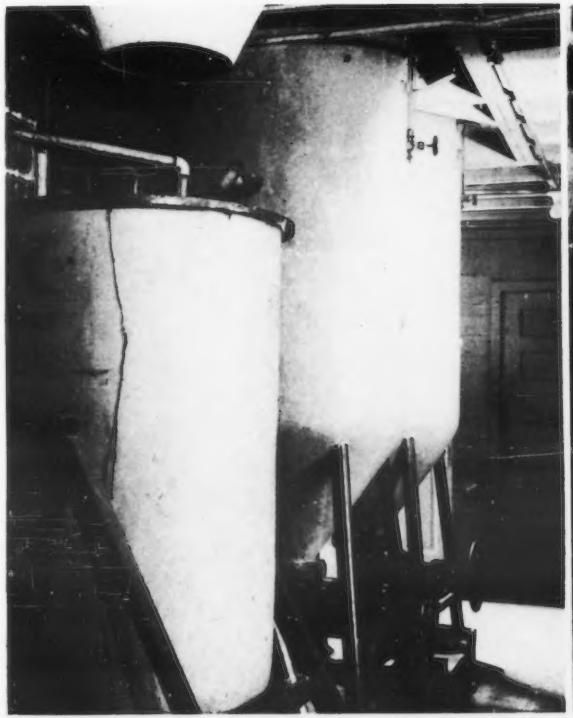

FIG. 3.

FIG. 4.

FIG. 3. *The three cookers; the open cooker in the foreground and the pressure cookers in the background.* FIG. 4. *The six-cell flotation cell showing the well formed froth.*

TABLE I

EFFECTS OF WATER EXTRACTION, ALKALI CONCENTRATION, STEAM PRESSURE, AND COOKING TIME ON AGGLOMERATION

Water extraction time, min.	Cooking conditions			Cooked wet wt.* from 500 gm. dry leaves, gm.	Yield of gum, dry wt., gm.	Gum analysis		
	Steam pressure, lb.	Time, hr.	Alkalinity, %			Acetone soluble, %	Toluene soluble, %	Detritus, %
0	10	2	0.75	2060	4.0	42.5	19.0	38.5
0	10	2	1.00	2086	1.7	39.1	17.6	43.3
0	10	2	1.25	1516	18.2	41.9	32.7	25.4
15 and 5	10	2	0.75	1410	Emulsified	—	—	—
15 and 5	10	2	1.00	1526	24.2	39.8	35.8	24.4
15 and 5	10	2	1.25	1229	19.7	41.4	37.0	21.6
0	10	3	0.75	2063	Emulsified	—	—	—
0	10	3	1.00	1526	Emulsified	—	—	—
0	10	3	1.25	1874	7.2	44.1	33.0	22.9
15 and 5	10	3	0.75	2147	Emulsified	—	—	—
15 and 5	10	3	1.00	1734	7.6	45.3	35.2	19.5
15 and 5	10	3	1.25	1559	6.6	45.6	29.8	24.6
0	10	4	0.75	1734	Emulsified	—	—	—
0	10	4	1.00	1567	4.5	46.8	22.4	30.8
0	10	4	1.25	1247	19.2	45.7	32.8	21.5
15 and 5	10	4	0.75	1526	12.4	41.9	30.5	27.6
15 and 5	10	4	1.00	1671	24.8	41.7	37.5	20.8
15 and 5	10	4	1.25	1287	18.7	40.4	43.4	16.2
0	40	2	0.75	1529	Emulsified	—	—	—
0	40	2	1.00	1568	1.0	48.3	24.1	27.6
0	40	2	1.25	1153	2.5	43.9	30.1	26.0
15 and 5	40	2	0.75	1452	10.6	44.1	33.3	22.6
15 and 5	40	2	1.00	1233	5.3	45.3	30.4	24.3
15 and 5	40	2	1.25	1449	5.5	45.3	32.1	22.6
0	40	3	0.75	1205	Emulsified	—	—	—
0	40	3	1.00	754	1.5	41.2	17.9	40.9
0	40	3	1.25	843	8.7	40.0	31.2	28.8
15 and 5	40	3	0.75	625	9.4	42.0	30.2	27.8
15 and 5	40	3	1.00	597	7.4	42.1	29.0	28.9
15 and 5	40	3	1.25	445	6.0	42.8	34.1	23.1

** This weight is lower than other laboratory data because the filtering was done on a large coarse filter and the leaves were relatively low in resin and rubber.*

spinach. For the most part, the slurry dropped loose from the filtering medium during rotation so that a new surface was continually available for filtration. Application of a sharp spray of water on the reverse side of the cheesecloth removed particles that were still adhering to the surface. This operation reduces the pH to 11.2–11.8. Subsequent flotation requirements necessitated a pH lower than 11.5 if excessively watery concentrates were to

be avoided. Consequently, leaves with a higher pH than 11.5 required a second pass through the trommel filter.

It was necessary to filter the cooked leaves immediately. On cooling and standing, an unidentified substance precipitated which tended to increase the difficulty of subsequent gum agglomeration. This difficulty (illustrated in Table II) was particularly marked for agglomeration without flotation.

TABLE II

EFFECT OF DELAY IN THE FILTERING PROCEDURE ON THE YIELD OF GUM

Cook	Filtration	pH	Milling	Yield gum, wet wt., gm.
Identical for all three runs	Filtered immediately hot	9.75	Milled at once after washing	4.0
Three-hour cook in 1½% NaOH solution (equivalent to a two hour cook at 10 lb. pressure) (3)	Filtered immediately hot	9.85	Milled 40 hr. after washing	4.0
	Filtered after standing for 48 hr.	9.60	Milled at once after washing	Emulsified

All three samples were cooked in the same way but one was left standing to cool in its alkaline liquor for 48 hr. while the other two were filtered and washed immediately. One of these was milled at once and the other was left standing for 40 hr. before milling. Both yielded 4 gm. of gum; this showed that once the leaves are filtered and washed they need not be milled immediately. However, the sample that was allowed to stand for 48 hr. before filtering and washing yielded no gum.

Milling and Screening

Two porcelain-lined pebble mills were used to comminute the leaves. One of the mills is a Vulcan amalgam barrel 36 in. diam. and 48 in. long; the other is a Straub, rib-cone mill 36 in. diam. by 36 in. long, with hollow trunions. The latter was used as a batch mill although suitable for continuous operation. The pebbles used were 1¾ to 2 in. Saskatchewan flint pebbles.

Since the gum was more easily agglomerated from the flotation concentrate these mills only comminuted the leaves, in contrast to the laboratory procedure where one pebble mill did both the work of comminution and agglomeration (2). While these mills ground the leaves rapidly they also started initial agglomeration. A decreased pH tended to aid agglomeration (Table III), and slurry consistency, pebble size, and number of revolutions per minute of the mill all affected it.

Cooked milkweed leaves were ground at a slurry consistency of 5% solids, milled for 3½ hr., and then put over a 60 mesh vibrating screen. The oversize comprised about one-seventh of the total bulk and had approximately the same acetone and benzene extracts as the remainder of the slurry. While these

TABLE III

EFFECT OF pH ON THREE SEPARATE BATCHES OF WASHED COOKED LEAVES ON GUM YIELD FROM 200 GM. OF MILKWEED LEAVES

pH	Yield gum, wet wt., gm.	Gum analysis		
		Acetone soluble, %	Toluene soluble, %	Detritus, %
9.8	8	36.5	27.2	36.3
6.8	6	39.4	24.2	36.4
6.3	6	—	—	—
4.8	17	45.6	25.3	29.1
9.6	4	41.7	20.6	37.7
5.9	20	47.9	23.5	28.6
9.6	15	31.5	29.6	38.9
4.8	19	48.0	21.5	30.5

mills did not produce complete agglomeration of the gum, they did agglomerate as much as one-quarter of the total gum into worms $\frac{1}{4}$ in. long. Therefore if the leaves were milled too long, the oversize would become enriched in gum. In a typical four hour run the percentage of resin and rubber in the oversize was approximately doubled. On the other hand, with a three hour grind approximately one-quarter of the bulk was retained in the oversize. Consequently a $3\frac{1}{2}$ hr. grind was adopted. This milled screened material was then read for the flotation cell.

Flotation and Thickening

The milled slurry had its resin–rubber fraction concentrated in the froth produced by a 6 cell Denver Sub A flotation cell (Fig. 4). The slurry contained its own frothing and collecting reagents. The frothing properties depended on the pH to which the cooked leaves were washed: the higher the pH the more watery and voluminous the froth became.

The pH of the concentrate was lower than that of the tails, as shown by the following pH measurements:

	pH	
Concentrate	8.6	9.3
Tails	9.1	9.7

Table IV illustrates the type of separation effected in a flotation run. The flotation heads were fed to Cell 2. The concentrates from Cells 2 and 3 were fed to Cell 1, and the concentrates from Cells 4, 5, and 6 were returned to Cell 2 again. Thus the final concentrate was withdrawn from Cell 1 and the final tails withdrawn from Cell 6.

The concentrate from the cell was watery and therefore was thickened before final agglomeration. There are two major reasons for this thickening operation. First, the gum agglomerates much more rapidly out of a thicker slurry, and second, a greater charge of concentrate (on a dry weight basis)

TABLE IV

FLOTATION CELL RUN

Cell	Heads			Concentrate			Tails		
	Solids, %	Acetone soluble, %	Toluene soluble, %	Solids, %	Acetone soluble, %	Toluene soluble, %	Solids, %	Acetone soluble, %	Toluene soluble, %
First	6.0	22.5	8.9	4.7	29.6	19.5	0.9	6.1	0.7
Second	3.7*	8.7*	0.9*	5.9	21.1	5.3	2.1	8.2	1.0
Third	2.1	8.2	1.0	6.0	23.8	12.3	1.7	5.3	0.7
Fourth	1.7	5.3	0.7	5.6	20.6	13.8	1.7	6.0	0.6
Fifth	1.7	6.0	0.6	4.3	10.0	0.6	1.7	2.7	0.7
Sixth	1.7	2.7	0.7	5.1	17.8	12.2	1.7	3.2	0.4
Over-all	3.7	8.7	0.9	4.7	29.6	19.5	1.7	3.2	0.4

** Not true analysis as that is head analysis only and the actual feed = head and concentration from Cells 4, 5, and 6.*

may be put into the agglomerating mill. This thickening was done by permitting the concentrate to settle for about six hours and then siphoning off the clear supernatant liquor. Agglomeration may be carried out immediately or after standing.

Agglomeration and Drying

Gum was agglomerated in a procelain lined Abbé pebble mill 30 in. diam. and 36 in. long, half filled with 1½ in. Saskatchewan flint pebbles. The thickened concentrate, either hot or cold, was added to the mill until the pebbles were just covered. Hot concentrates agglomerated much more rapidly and the gum showed no variation on analysis from gum obtained from a cold concentrate. Eight to ten hours of milling were required to agglomerate a cold concentrate into pea-sized gum pieces whereas near the boiling point of water the gum agglomerated in four to five hours. When the gum had been agglomerated to this size the charge was put over a 60 mesh vibrating screen. The undersize was sent back to the flotation cell and the oversize was returned into the agglomerating mill. The mill was half filled with hot water and rolled for one-half hour. At the end of this time the gum had agglomerated into one or two pieces (Fig. 5).

The gum contained from 20 to 35% of acetone- and toluene-insoluble materials termed detritus. Forty per cent of this was ash consisting for the most part of ground flint and porcelain. Experiments have shown that ground porcelain does not hinder gum agglomeration. The detritus content may possibly be reduced by modification in procedure and equipment, such as lining the mills with wood.

The gum was then creped on rubber washing rolls, spread on screen trays, and dried in an air or vacuum oven. After three to four days in the air-drying oven the initial 15 to 25% moisture was reduced to less than 1%. The equilibrium moisture content at a relative humidity of 30 to 40% was approximately 2%.

FIG. 5. *Samples of crude gum as removed from the agglomerating pebble mill.*

Acknowledgments

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

I. FERMENTATION OF WHEAT MASHES BY *AEROBACILLUS POLYMYXA*[1]

By G. A. Ledingham[2], G. A. Adams[2], and R. Y. Stanier[2]

Abstract

Isolation of *Aerobacillus* strains with desirable fermentative characteristics was facilitated by pasteurization of the original inoculum. Both cultural characteristics and fermentative capacity of the original isolates were extremely variable. Dissociation into more variant types occurred in later generations giving rise to further differences in fermentative characteristics.

In the preparation of mashes particle size of wheat is unimportant in relation to yield provided the kernel has been broken. Since prolonged cooking is harmful, a standard procedure of sterilization for 1 hr. at 121° C. has been adopted. Mashes containing over 15% wheat by weight are inefficiently fermented. Acid production in the mash may be controlled by the addition of excess calcium carbonate at the beginning of the fermentation, or by the addition of ammonia as required. The most satisfactory fermentation temperature is about 32.5° C.

The addition of yeast extract, malt extract, dried yeast, or corn steep liquor is essential for the preparation of an active inoculum. Fermentation of whole wheat mashes may also be enhanced by yeast extract. Removal of the gluten has little effect, but the bran, shorts, germ, and soluble nitrogenous constituents are necessary for a normal fermentation. Pure wheat starch with inorganic supplements can be only partially utilized.

Under anaerobic conditions fermentation of a 15% mash is complete in 60 hr., but continuous removal of the carbon dioxide reduces this time to 48 hr. The diol–ethanol ratio for anaerobic fermentations is of the order of 1.3 : 1.0. Aerobic conditions inhibit the fermentation, particularly ethanol formation, and 120 hr. are required for completion. The diol–ethanol ratio, however, may be raised to 3 : 1 or higher.

Introduction

The events following Pearl Harbor indicated an urgent need for four carbon compounds in the manufacture of synthetic rubber and other war chemicals required in substantial volume. At that time all channels for the use of surplus wheat were under examination and, naturally, the possibilities of utilizing fermentation processes were considered. Although the production of ethanol and butanol from grains and the conversion of these alcohols to butadiene was known from previous studies, it was felt that the fermentation of starchy products directly to four-carbon compounds might be advantageous, particularly with respect to the economy of critical materials. Information available in the literature suggested that, of the four-carbon compounds available, 2,3-butanediol offered the best possibilities provided that it could be produced in substantial volume. As work progressed and material became available for studying the properties of this compound, the information

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indicated that certain forms of the diol would be valuable for purposes such as anti-freeze. Consequently an investigation concerned with the production and properties of 2,3-butanediol was undertaken in the Division of Applied Biology; the results will be reported in a series of papers of which this, the first, deals primarily with methods and fermentation problems.

When this investigation was initiated there were two bacterial fermentations known that yielded substantial amounts of 2,3-butanediol. The most information was available on the *Aerobacter* fermentation, first investigated early in this century by Harden and Walpole (3) and later by Scheffer (9). More recent studies by Fulmer, Christensen, and Kendall (2) in Iowa State College had raised the yields to a point where they had industrial possibilities. This fermentation had been shown to yield a mixture of two optically active forms of the diol, consisting of the *d*-and *meso*-form, with a preponderance of the latter, and in addition a variable but usually small amount of ethanol. It could be carried out only on sugars since *Aerobacter* species generally have very weak diastatic powers.

Prior to 1942 little work had been done on the other 2,3-butanediol fermentation (by *Aerobacillus polymyxa* (Prazmowski) Donker) apart from the pioneering investigations by Donker (1), which for the first time revealed the nature of the carbohydrate dissimilation by this species. Kluyver and Scheffer appear to have made a few large scale experiments on the fermentation of starchy substrates by *A. polymyxa* as mentioned in their patent (6) but no extensive studies on this problem had been made and fermentations on a commercial scale had never been undertaken. *A. polymyxa* is actively diastatic, and since the fermentation of wheat mashes to diol was the immediate objective further work with this organism appeared most attractive. Later, when the 2,3-butanediol produced was found to be the pure *l*-isomer with anti-freeze properties not shared by the *meso*-form, its production became even more essential.

During the course of these investigations a third fermentation, by *Aeromonas hydrophila*, in which an *l-meso* mixture of 2,3-butanediol was produced, was described by workers from these laboratories (10). The organism, like *Aerobacter*, lacked diastatic properties and hence had to be grown on hydrolysed mashes or sugar solutions. Other than the results of these preliminary investigations little information is yet available concerning it.

Since this project was initiated early in 1942 a great deal of work has been done elsewhere on both the *Aerobacter* and *Aerobacillus* fermentations. Investigations initiated simultaneously in the United States made possible a co-operative plan not contemplated at the outset. The Northern Regional Research Laboratories of the United States Department of Agriculture in Peoria, Ill., called conferences of all investigators interested in this field. Through these conferences, the pooling of reports and the exchange of cultures, progress on the problem was greatly expedited. We wish to acknowledge the substantial aid received from this institution. In addition valuable assistance and advice has been received from Joseph E. Seagram & Co., Louisville, Ky.;

Distillers Corporation, LaSalle, Que.; and the Department of Biochemistry, University of Wisconsin, Madison, Wis.

In Canada several different phases of the problem were undertaken elsewhere concurrently with our own studies. Thus certain biological problems in both the *Aerobacter* and *Aerobacillus* fermentations have been supported by the Associate Committee on Grain Research at the University of Alberta. Other aspects were studied by the Division of Dairy Science and Bacteriology of the Department of Agriculture in Ottawa, and the conversion of the diol to butadiene was studied by the Division of Chemistry of these laboratories.

Studies on the Organism

Isolation of Aerobacillus polymyxa

Aerobacillus polymyxa may be isolated from a wide variety of natural sources such as soil, soil water, and fresh and decaying plant materials. Rich garden soil is one of the most dependable sources. It has been found that pasteurization of the original inoculum is very important. In a few preliminary isolations unpasteurized inocula were used with the result that the aerobacilli were frequently overgrown in the enrichment medium by non-spore-forming organisms. Furthermore none of these strains showed promise in later fermentation studies.

The isolation procedure finally adopted was as follows: the source material was shaken up with sterile water and 5 ml. of the resulting suspension was placed in a sterile test-tube and held in a water-bath at 80° C. for 10 min. The pasteurized suspension was then inoculated in 1 ml. amounts into tubes of either lactose or starch broth (1% peptone with 1% lactose or 1.5% starch) and incubated at 30° C. Enrichment cultures that showed gas after two to three days were streak plated. Of a variety of media used for streaking purposes, the most generally satisfactory was neutral red agar of the following composition.

Starch or lactose	20 gm.
Peptone	10 gm.
Yeast extract	5 gm.
Neutral red	0.05 gm.
Agar	15 gm.
Water	1000 ml.

This medium has several advantages: *A. polymyxa* grows vigorously on it; the colonies of this species are usually pink or red in colour, which is true of very few other organisms found in the enrichment cultures; and the neutral red serves as an indicator for acid production. However, when one has become acquainted with the various colony types under which *A. polymyxa* is capable of masquerading, a plain starch–peptone or starch–yeast agar is just as satisfactory.

Recognition of *A. polymyxa* colonies is rendered difficult because of their extreme variability. Even on first plates the organism may appear in any of at least half a dozen colony forms each of which might be taken by the

uninitiated for a separate species (Plate I). In addition to the pink or red coloration of the colonies on neutral red agar mentioned above, several other characteristics have been noticed that are useful in making rapid decisions as to whether or not a given culture belongs to the *Aerobacillus* group. Outstanding are the pleasant fruity odour so characteristic of these organisms, the manner in which colonies producing a thick slime become blown up by gas bubbles, and the rapidly spreading growth on moist plates. Even so, the differences in colony form, consistency, surface, and elevation that may occur in different isolates are so great that much experience is needed to recognize these organisms with certainty.

In all, 100 strains of *A. polymyxa* were originally obtained for comparative studies. Sixty of these were isolated in these laboratories from pasteurized material and prior to this about two dozen strains had been isolated from unpasteurized soil. Further strains were obtained from other laboratories: two from the Northern Regional Research Laboratories at Peoria, Ill., 10 from Dr. N. R. Smith of the U.S.D.A., Washington, D.C., five from the Department of Field Crops, University of Alberta, and one from the Schenley Research Institute, Laurenceburg, Ind. At the present time, owing to the dissociation of most of these cultures into numerous variants, several hundred cultures are now available and continuous selection of the best strains is being carried on.

Stock cultures in active use may be carried on agar slants containing 5% whole wheat or 2% soluble starch plus 0.5% yeast extract and 0.5% calcium carbonate. Slants should be prepared several days before use in order that the surface may be allowed to dry before inoculation, otherwise splitting and blowing of the agar will ensue. Owing to the marked tendency of the organism to dissociate it is unwise to rely wholly on agar slants to carry different strains indefinitely. All cultures have therefore been preserved in vacuum tubes by the lyophile method.

Morphological and Biochemical Characteristics

The morphological and biochemical properties of *A. polymyxa* have been described in a number of publications, the most recent of which is that of Porter, McCleskey, and Levine (8); consequently the results of our own investigations on this phase will be described very briefly.

The morphology of all the strains that we have examined is much the same. Young vegetative cells are plump rods, very actively motile by means of peritrichous flagella. They are Gram-negative and measure approximately 1.0 by 4.0 to 8.0 μ. The vegetative cells undergo rapid autolysis as cultures age, even when spore formation does not occur. Spores are elliptical and terminally or subterminally located in the cell. Spore formation takes place after two to three days at 30° C. under aerobic conditions but it is delayed and less extensive under a restricted oxygen supply. When recently isolated, cultures produce spores in great abundance but maintenance under laboratory conditions leads to a lessening of this ability. Many of the cultures received from N. R. Smith were asporogenous although their other characteristics left

no doubt as to their proper designation. We have since noticed a tendency for a similar condition to arise in our own cultures.

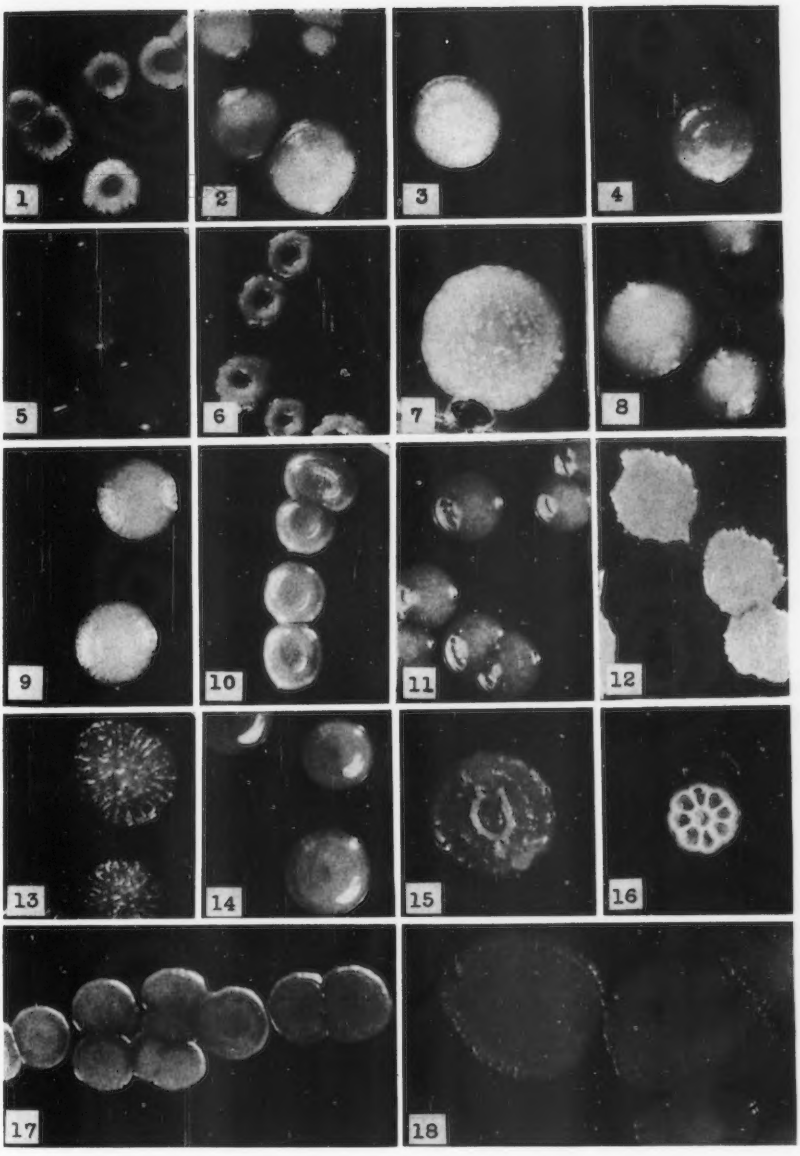
The biochemical characteristics of the strains studied are remarkably uniform. The organisms grow vigorously under both aerobic and anaerobic conditions in peptone or yeast extract media containing a fermentable carbohydrate. In the absence of a suitable carbohydrate, growth does not occur under anaerobic conditions and, even under aerobic conditions, is fairly slight. The nitrogen and growth factor requirements are complex. Fermentative properties as determined by gas formation in deep agar tubes are almost identical for all strains. The 27 strains tested (including *N.R.R.L.* 510, a number of N. R. Smith's cultures, and many of the isolates made in this laboratory) ferment arabinose, xylose, glucose, galactose, levulose, mannose, lactose, sucrose, maltose, cellobiose, raffinose, trehalose, starch, inulin, sorbitol, and salicin but not dulcitol or inositol. The only sugar that was found not to be fermented by all strains was rhamnose, which was fermented by approximately half of the organisms tested. All strains are *V.P.* and catalase positive, liquefy gelatine, and reduce nitrates to nitrites. The only difference between these results and the findings of Porter, McCleskey, and Levine lies in the fermentation of rhamnose; Porter, McCleskey, and Levine found that none of the *A. polymyxa* strains that they studied were able to attack this compound.

Methods

Mash Preparation

In the laboratory, fermentations have always been carried out on 300-ml. amounts of medium in 500-ml. Erlenmeyer flasks. In all cases, except where the influence of pH or carbonates was being tested, 1% calcium carbonate was added. Mash percentages are based on the weight of grain in the final volume of mash; i.e. a 15% mash is one containing 15 gm. of grain in 100 ml. of mash.

Our methods of mash preparation have varied from time to time. In the beginning the desired amount of grain was simply weighed into flasks and the volume was made up to 300 ml. with water. However, mashes so prepared, particularly at high concentrations, proved somewhat unsatisfactory owing to the tendency of the grain to settle out and cook to a hard cake during sterilization, with subsequent irregular fermentation. As a result the procedure of preparing mashes in bulk and cooking them lightly with constant agitation to ensure an even dispersal of the grain before dispensing them into flasks was adopted. For the past 18 months all mashes have also been preliquefied by treatment, for 10 min. at 70° C., with malt in amounts equivalent to 1% of the weight of grain. This treatment aids greatly in stirring and the distribution of the inoculum. Sterilization of the flasks of mash has been carried out at 15 lb. pressure for 60 to 80 min.; the autoclave is equipped with a compressed air line, which permits maintenance of the pressure at 15 lb. per sq. in. during cooling and thus prevents boiling over of the medium in the flasks—a danger if high concentrations of starch are sterilized in the customary manner.



Aerobacillus polymyxa strains on neutral red agar, illustrating some of the different colony types that occurred at the time of isolation: Fig. 1 (C3); Fig. 2 (C4); Fig. 3 (C8); Fig. 4 (C14); Fig. 5 (C16); Fig. 6 (C17); Fig. 7 (C18); Fig. 8 (C19); Fig. 9 (C44); Fig. 10 (C42); Fig. 11 (C45); Fig. 12 (C48); Fig. 13 (C51); Fig. 14 (C52); Fig. 15 (C56); Fig. 16 (UA 233b); Fig. 17 (N.R.R.L. 510); Fig. 18 (N.R.R.L. 510. Rough).

Each flask, brought beforehand to a temperature of 30° C., was inoculated with 10 ml. of a 24-hr.-old culture in a liquid starch medium. The inoculation medium consisted of:

Soluble starch	2%
Yeast extract	0.5%
Calcium carbonate	1%

Unless otherwise stated fermentations were always carried out at 30° C.

Analytical Procedures

At the conclusion of an experiment the fermented mashes were centrifuged and analyses were run on the supernatant liquid. Analyses were always made for butanediol and ethanol; in addition the pH, the titratable acidity, and the acetoin concentration were at times determined. The figures for butanediol and ethanol presented in the subsequent papers in this series are always expressed as percentages by weight in the mash following fermentation. In some cases the percentage of 'total products' formed, i.e. the sum of the figures of diol and ethanol have been given. Needless to say other products (acetoin, acetic acid, carbon dioxide, hydrogen) are also formed but they are not included in the heading of 'total products.' Where ratios between butanediol and ethanol are given they refer to the weight percentage of butanediol over weight percentage of ethanol.

Throughout the early stages of this study many changes were made in the methods of estimating butanediol in fermented mashes. Such slow and inaccurate methods as the gravimetric determination of diol in ether extracts of the mash soon gave way to more rapid chemical methods. During much of the work reported in this series the following procedure was used. An aliquot portion of the clear fermented mash was distilled in the presence of an excess of a high boiling fraction of kerosene (200° to 220° C.). The aqueous phase of the distillate contained all the diol and could be analysed directly by a periodate oxidation method* developed in the Department of Biochemistry, University of Wisconsin. The method is based on the oxidation of each mole of 2,3-butanediol by periodic acid to yield two moles of acetaldehyde, which is absorbed in bisulphite. The aldehyde bisulphite compound is decomposed by alkali and the bisulphite titrated with dilute standard iodine solution. Acetoin, which is present to a small extent in the fermented mashes, interferes quantitatively and a correction for it must be made.

Since the kerosene distillation procedure did not always give consistent results, it has been succeeded by a butanol extraction in which the fermented mash was previously saturated with potassium carbonate. The diol in the butanol extract was then estimated by the acetaldehyde titration or by a modified periodate oxidation method in which the unused periodate is estimated in the presence of iodate by iodometric titration at a pH of 6.0 to 7.0 (11).

* *Description unpublished.*

The estimation of acetoin was made on the original fermentation solution by an unpublished method outlined by the Northern Regional Laboratory, Peoria, Ill.; it was based on the oxidation of acetoin, by ferric chloride, to diacetyl, which was converted by reaction with hydroxylamine to its dioxime and precipitated as the nickel salt. The nickel salt was filtered off, decomposed by acid, and the liberated hydroxylamine was oxidized by ferric iron; the ferrous iron formed in the reaction was determined by titration with potassium permanganate. The method is subject to a correction factor, as quantitative formation of nickel dimethyl glyoxime is not realized. In later work the method of Langlykke and Petersen (7) was found to be superior to the one described above and it was used exclusively.

Following its fractional distillation from the fermented mashes ethanol was determined by dichromate oxidation.* The distillation fractionated the ethanol and butanediol completely; however, a small proportion of the acetoin distills over with the ethanol and a correction factor must be applied. In most mashes the proportion of acetoin present is so small that the correction factor is negligible.

Calculation of Theoretical Yields

In a few places reference has been made to the 'theoretical yield' of butanediol and ethanol resulting from complete fermentation of a given mash concentration. Naturally it is impossible to arrive at as satisfactory a theoretical yield for so complex a fermentation as for the alcoholic or homofermentative lactic acid fermentations. In calculating the theoretical yield the following assumptions have been used: (i) that apart from butanediol and ethanol no other organic end products occur in appreciable amounts; (ii) that each molecule of hexose yields two molecules of a C_2 compound and two molecules of a C_1 compound (carbon dioxide); (iii) that butanediol is formed by condensation, and ethanol by reduction, of the C_2 compound. Thus two molecules of C_2 compound will yield one molecule of butanediol or two molecules of ethanol.

Since the molecular weight of ethanol (46) is almost exactly half that of 2,3-butanediol (90) it follows that from a given weight of C_2 compound and hence of hexose the weights of butanediol and ethanol produced would be essentially the same. Thus for purposes of calculation on a weight basis these two compounds may be treated as one ('total products'). The total products that one can expect to be formed from a given weight of hexose should, on the basis of the above assumptions, weigh half as much as the hexose fermented. Since the sugar derived from the hydrolysis of starch weighs 10% more than the original starch, a factor of 10% must be included for fermentations of starch mashes. As an illustration of the method of calculation the theoretical yield is derived for a 15% wheat mash made from wheat with a starch content of 55%. This mash would contain 8.25% of starch, which yields on hydrolysis 9.07% of glucose, from which one can

* *Unpublished method, Department of Biochemistry, University of Wisconsin, Madison, Wis.*

expect a theoretical yield amounting to 4.53% of total products. In actual fact small amounts of acetoin and acetic acid are always produced so that even under optimum conditions the 'theoretical yield' as calculated above can never be obtained.

Owing to the marked fluctuations that occur in the butanediol–ethanol ratio, it is not possible to calculate a theoretical yield of butanediol alone.

Factors Affecting the Fermentation

In the development of any new industrial fermentation where the potentialities of the organism involved are relatively unknown, there are many factors that must be considered. A detailed description of the studies on certain problems will receive attention in later papers of this series; only a generalized account is presented here. The various factors involved may be broadly classified under three headings as follows: (i) those relating primarily to the organism, particularly the native fermentative ability of the different strains and the provision of an inoculum of dependable maximum fermentative capacity; (ii) provision of a substrate that is ideal as a source of fermentable carbohydrates and nutritive factors; and (iii) provision of an environment that is conducive to the rapid production of the desired end-products.

Fermentative Capacity of Different Strains

The production of 2,3-butanediol and ethanol by different strains on 15 and 25% whole wheat mashes after 3- and 10-day periods, respectively, is shown in Table I. There are very great differences in the amount of 2,3-butanediol and ethanol produced, depending on the strain of *Aerobacillus* used. Out of 54 cultures tested on the 15% mash, 21 produced more than 3.0% total products in three days. On the 25% mash 13 cultures produced over 5% total products, but in spite of their prolonged fermentation time, which was given to indicate maximum fermentative capacity, in none was the fermentation as near completion in ten days as on the 15% mash in three days. It is also apparent from the table that certain strains were equally poor on both mashes. On the other hand, certain strains that were only moderately good on the three day fermentation were relatively much better at the longer period. This effect may well be due to the ability of certain strains to tolerate higher concentrations of end-products. While there were a few marked variations in the diol–ethanol ratio, in general it remained constant. The analysis of variance on the results indicated that the ratio for cultures grown on the 25% mash did not differ significantly from that of cultures on the 15% mash.

Approximately one year after these basic analyses had been carried out further tests were made on the yield of diol and ethanol from 70 different variants of these original strains. Part of these results are shown in Table II. Some of the cultures had produced as many as six different variants and by comparison with the original cultures it may be seen that in certain instances new variant strains were superior to the original, although others were

TABLE I

COMPARATIVE YIELDS OF 2,3-BUTANEDIOL AND ETHANOL BY ORIGINAL STRAINS OF *Aerobacillus polymyxa* GROWN ON WHOLE WHEAT MASHES

2,3-Butanediol + ethanol, %	Culture number	
	3-Day fermentation on 15% mash†	10-Day fermentation on 25% mash††
Over 5.5		*C*2, *C*5, *C*12, *C*38
5.5 – 5.0		*C*1, *C*2*a*, *C*6, *C*9, *C*14, *C*17, *C*21, *C*42, *UA*233*a*
5.0 – 4.5		*C*7, *C*10, *C*13, *C*18, *C*20, *C*23, *C*24, *C*25, *C*26, *C*27, *C*29, *C*37, *C*39, *C*44, *C*48, *C*51, *C*53, 510, 510*R*, *UA*233*b*
4.5 – 4.0		*C*3, *C*22, *C*31, *C*36, *C*40, *C*41, *C*46, *C*49, *C*55, *UA*206
4.0 – 3.5	*C*18, *C*38, *UA*233*b*	*C*19, *C*33, *C*34, *C*54, *UA*211
3.5 – 3.0	*C*2, *C*2*a*, *C*5, *C*6, *C*7, *C*13, *C*20, *C*21, *C*22, *C*23, *C*25, *C*42, *C*47, *C*48, *C*49, *C*51, 510, 510*R*	*C*8, *C*52, *UA*24*a*
3.0 – 2.5	*C*12, *C*14, *C*17, *C*24, *C*27, *C*29, *C*31, *C*40, *C*53, *UA*233*a*	*C*35
2.5 – 2.0	*C*1, *C*3, *C*9, *C*10, *C*11, *C*33, *C*34, *C*39, *C*41, *C*46, *C*52, *C*55, *UA*206	*C*11, *C*28
Below 2.0	*C*8, *C*19, *C*26, *C*28, *C*35, *C*36, *C*37, *C*54, *UA*24*a*, *UA*211	

† *Theoretical yield of butanediol + ethanol = 4.5%.*
†† *Theoretical yield of butanediol + ethanol = 7.5%.*

decidedly inferior. Culture *C*8, which was originally a very poor one from the standpoint of product yield, has now produced several variants that are moderately good diol producers. This strain received considerable attention during the past year because of the fact that it was resistant to all strains of bacteriophage so far encountered (5). It is interesting to note that a poor strain may carry potentialities that will be useful provided variants are selected from it.

Inoculation Medium

The production of bacterial cells rather than the end-products of fermentation is the main consideration in the preparation of inoculum. However, factors other than cell count must be considered since the numbers present may vary over wide limits without affecting the fermentation. Whole wheat mash (5% whole wheat + 1% calcium carbonate) makes rather a sluggish inoculation medium in spite of the fact that direct counts after growth for 24 hr. indicate the presence of approximately 200 million cells per cc. Addition

TABLE II

COMPARATIVE YIELDS OF 2,3-BUTANEDIOL AND ETHANOL BY CULTURAL VARIANTS OF *Aerobacillus polymyxa* ON 15% WHOLE WHEAT MASH FERMENTED FOR THREE DAYS

Original culture No.	No. of variants	Yield, 2,3-butanediol + ethanol, %					
		3.5 – 4.0	3.0 – 3.5	2.5 – 3.0	2.0 – 2.5	1.5 – 2.0	1.0 – 1.5
*C*2	3	*C*2(3)	† *C*2(1)	*C*2(2)			
*C*3	6	*C*3(1) *C*3(2) *C*3(3) *C*3(6)	*C*3(5)		†	*C*3(4)	
*C*5	3	*C*5(3)	†	*C*5(2)			*C*5(1)
*C*6	5		† *C*6(2) *C*6(5)	*C*6(3) *C*6(4)	*C*6(1)		
*C*8	4		*C*8(3) *C*8(4)	*C*8(2) *C*8(5)			†
*C*20	4	*C*20(3) *C*20(4)	*C*20(2) †				*C*20(1)
*C*23	3		† *C*23(3)	*C*23(2)	*C*23(1)		
*C*42	4		†	*C*42(1) *C*42(3)	*C*42(2) *C*42(4)		
*C*53	4	*C*53(1)		† *C*53(2) *C*53(4)	*C*53(3)		
*UA*233*a*	4	*UA*233*a*(1) *UA*233*a*(4)	*UA*233*a*(3) *UA*233*a*(5)	†			
*UA*233*b*	3	† *UA*233*b*(3)	*UA*233*b*(1) *UA*233*b*(2)				
*UA*211	2		*UA*211(1)			†	

† = *Yield of original isolates (see Table I).*

of 0.5 to 1.0% yeast extract will increase these numbers fourfold and make a very much more active inoculation medium. For laboratory use, where pipetting is essential, the whole wheat may be replaced with 2.0% soluble starch.

The addition of yeast extract to the inoculation medium would be quite expensive for use in a commercial fermentation process, hence other substitutes are being investigated in connection with our pilot plant operations. Very satisfactory results have been obtained using malt extract, malt sprouts, corn steep liquor, or dried yeast. Comparison of these, as well as other supplements with yeast extract, is now being made.

In the laboratory a number of experiments have been carried out with the soluble-starch–yeast-extract medium to determine the optimum amount and age of the inoculum to be added to the fermentation medium. The results of tests carried out with strain *C*38(1) grown for periods varying from 10 to 48 hr. are shown in Table III. Analyses for products of fermentation were

TABLE III

EFFECT OF AGE OF INOCULUM ON PRODUCTION OF 2,3-BUTANEDIOL AND ETHANOL BY *Aerobacillus polymyxa C*38(1)

Mean values

Age of inoculum, hr.	Fermentation time, hr.			
	48		72	
	Butanediol, %	Ethanol, %	Butanediol, %	Ethanol, %
10	2.09	1.18	2.21	1.36
16	2.04	1.16	2.29	1.40
24	2.12	1.28	2.28	1.37
48	2.04	1.22	2.26	1.38

Analyses of variance

Source of variance	Butanediol		Ethanol	
	D.f.	Mean square	D.f.	Mean square
48-hr. fermentation				
Age of inoculum	3	0.0035	3	0.0086
Residual (error)	8	0.0023	7	0.0067
72-hr. fermentation				
Age of inoculum	3	0.0033*	3	0.00063
Residual (error)	8	0.00064	8	0.00060

* *Exceeds mean square error, 5% level of significance.*

made at 48 and 72 hr. intervals after inoculation with 10 cc. of the inoculum in 300 cc. of mash. At the 48 hr. fermentation level, no significance could be attached to the age of the inoculum, in so far as the production of either butanediol or ethanol was concerned. However, at the 72 hr. fermentation level, production of butanediol was significantly less for the 10 hr. inoculum: from practical considerations, this result is deemed to be fortuitous. Further work is required covering a wider range of growth periods and actual cell counts in order to clarify this question. Similar results were obtained when the amount of inoculum was varied. No difference in the fermentation results were obtained using as little as 1% or up to 30% of the mash volume as inoculum. On the whole, experiments with the inoculum have shown that, provided an active culture is obtained, quantity or age of inoculum are not critical factors within wide limits.

Fermentation Medium

Wheat was chosen as the fermentation medium for this investigation because any wartime industrial development of the process in Canada would certainly require its use. Furthermore, the complex nutritional requirements of *Aerobacillus polymyxa* are most readily met through the use of a whole grain cereal. It has been recognized however, that there may be advantages in carrying out a physical separation of the different components and subsequently fermenting a more highly purified starchy concentrate.

Almost all the experiments carried out in this investigation were made using No. 3 Northern Marquis wheat of 56% starch content. At this stage no attempt was made to assess the effect of factors such as grade or variety. The factors studied here include effect of grind, effect of cooking time and temperature, concentration of mash, pH control, and the nutritional role played by the different constituents of the grain and the effects of supplementary nutrients.

Effect of Grind

Coarse grinding usually facilitates feed recovery, particularly where feeds are removed by screening. However, coarse particles may decrease the availability of the starch to the organism and reduce yields and efficiency of the fermentation. In order to determine whether these were important considerations, three portions of a well-mixed sample of grain were ground to give coarse, medium, and fine meal of sieve analysis shown in Table IV.

TABLE IV

SIEVE ANALYSIS OF DIFFERENT GRINDS OF WHOLE WHEAT

Percentage of wheat	Coarse	Medium	Fine
Retained on 20 mesh	55.7	41.1	00.0
Retained on 40 mesh	27.9	38.6	75.4
Retained on 60 mesh	4.7	8.2	12.8
Retained on 80 mesh	1.9	3.6	2.8
Retained on 100 mesh	1.4	2.4	4.6
Passing 100 mesh	8.2	6.1	4.3

Yield data from 72- and 96-hr. fermentations with three different strains of *Aerobacillus* (*C*53 (1), *C*4(2), *C*3(2)) on mashes prepared from whole unground wheat and the above grinds were subjected to analysis of variance the results of which are shown in Table V. Butanediol and ethanol production were not significantly affected by the level of grind. However, lack of grinding did affect the yield of both products but in the case of butanediol the magnitude of this effect varied with the organism.

It may be seen from Table VI that on the average the ground samples produced more butanediol than whole wheat for all organisms. However, the reduction in butanediol yield attributable to lack of grinding was not

TABLE V

ANALYSES OF VARIANCE OF 2,3-BUTANEDIOL AND ETHANOL PRODUCTION BY *Aerobacillus polymyxa* FROM WHEAT OF DIFFERENT GRINDS

Source of variance	Degrees of freedom	Mean square	
		Butanediol	Ethanol
Grind	3		
Whole wheat vs. ground	1	3.3917**	1.9647**
Grinding	2	0.0214	0.0002
Grinding × organisms	6		
Whole wheat vs. grind × organisms	2	0.1058**	0.0157
Ground wheat × organisms	4	0.0054	
Grinding × time	3		
Whole wheat vs. ground wheat × time	1	0.1641**	0.0196
Ground wheat × time	2	0.0087	
Grind × time × organism	6	0.0080	0.0113**
Duplicate	24	0.0050	0.0010

* *Exceeds mean square error 5% level of significance.*
** *Exceeds mean square error 1% level of significance.*

TABLE VI

INTERACTION EFFECTS OF GRIND BY ORGANISM AND GRIND BY TIME ON THE 2,3-BUTANEDIOL YIELD (%)

Grind	Organism			Fermentation time (hr.)	
	*C*53(1)	*C*4(2)	*C*3(2)	72	96
	2,3-Butanediol, %				
Ground wheat	2.97	2.95	3.08	2.86	3.14
Whole wheat	2.58	2.24	2.34	2.11	2.66

equally manifested by all organisms, *C*53(1) being relatively more efficient in utilizing whole wheat. Beneficial effects of grind are noted for both fermentation periods. As might be expected, the disadvantageous effects of unground wheat are less evident for the longer fermentation period. The average ethanol production from ground samples was 1.82% and only 1.36% for whole wheat.

Effect of Cooking Time and Temperature

The grain mashes must be sterilized after malt liquefaction by a cooking or autoclaving treatment. Preliminary experiments indicated that prolonged cooking at high temperatures affected the *Aerobacillus* fermentation adversely. Experiments have therefore been conducted in order to establish the range of cooking treatments that are adequate from the standpoint of mash sterilization, and that at the same time do not reduce the yields of 2,3-butanediol

and ethanol. Fifteen per cent whole wheat mashes were subjected to a 10 min. malting treatment at 70° C. after which the mash temperature was raised quickly to 100° C. and maintained there for five minutes. Aliquots (300 ml.) were placed in 500 ml. Erlenmeyer flasks for cooking and fermentation. The samples were autoclaved for periods of 0.5, 1.0, and 3.0 hr. at temperatures ranging from 100° to 130° C. Fermentation of the mashes with strain *C3*(2) was then allowed to proceed for 72 hr. under conditions outlined elsewhere. The 2,3-butanediol and ethanol contents of the various fermented mashes are shown in Table VII.

TABLE VII

EFFECT OF COOKING TIME AND TEMPERATURE ON 2,3-BUTANEDIOL AND ETHANOL PRODUCTION BY *Aerobacillus polymyxa C3*(2) AFTER 72-HR. FERMENTATION ON 15% WHEAT MASH

Time of cooking, hr.	Temperature, ° C.					
	100	108.1	115.1	121.0	126.0	130.4
	2,3-Butanediol, %					
½	2.58	2.77	2.75	2.67	2.62	2.67
1	2.72	2.77	2.65	2.84	2.70	2.73
3	2.95	2.81	2.57	2.67	2.15	1.65
	Ethanol, %					
½	1.71	1.74	1.72	1.68	1.67	1.76
1	1.77	1.77	1.69	1.64	1.65	1.55
3	1.86	1.69	1.61	1.47	1.14	0.78

The results indicate that a rather wide range of cooking treatments may be employed without reducing the fermentation yield. Unquestionably, cooking for three hours at 126° C. and higher temperatures is harmful, leading to reduced yields of both ethanol and butanediol. Otherwise, with the exception of the yield after the three hour treatment at 100° C. the results are of the same order. The exceptional result is considered fortuitous since it has not been borne out by subsequent work. Sterile mashes were obtained consistently with all of the cooking treatments except that of 0.5 hr. at 100° C. On the basis of these experimental data, cooking for one hour at 121° C. has been adopted as a standard practice since it is well within the range satisfying all requirements.

Mash Concentration

In deciding on the mash concentration that should be used in an industrial fermentation a number of considerations are of importance. First of all the mash must not be so thick and viscous that it will be impossible to pump it or force it through the mash cooler. Other limiting factors relate to the efficiency of the fermentation. If the supply of fermentables is too high, frequently the organism will not be able to tolerate its own fermentation end-products. This retardation of fermentations on high mash concentrations

may be seen in Fig. 19. In this experiment, 5, 10, 12.5, 15, 17.5, 20, and 25% whole wheat mashes were fermented for 72 hr. by *Aerobacillus* strain *N.R.R.L.* 510. The 5 and 10% mashes were almost completely fermented in

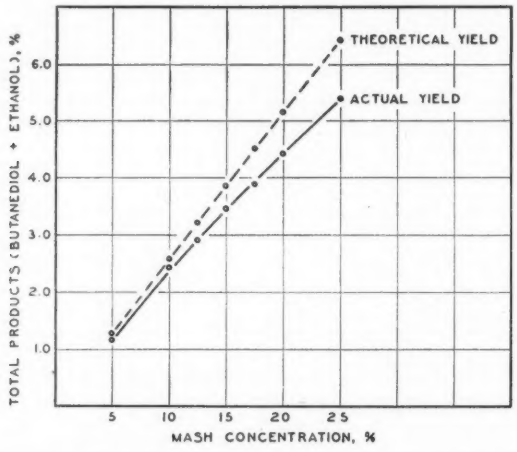


FIG. 19. *Comparison of actual with theoretical yield of 2,3-butanediol and ethanol on whole wheat mashes, three-day fermentations.*

three days but above this, while actual yields of product were greater, the efficiency of the fermentation was poorer. Increasing the length of fermentation time will not help materially for with 25% mashes there is only a very slight improvement even though the fermentation is allowed to proceed for 10 days. In general, mashes whose concentrations are over 15% are very thick and viscous and unless they are pre-liquefied it is very difficult to thoroughly mix in the inoculum.

The incomplete fermentation of mashes at high concentrations was a phenomenon that it seemed possible might be due to the accumulation of the end-products and their adverse effect on the organisms. Reference is made in the Kluyver patent (5) to the desirability of periodic removal of the alcohol in order to obtain a complete fermentation. On the whole this did not seem to have much practical value but nevertheless the effect of added ethanol on the fermentation of 10 and 20% wheat mashes was studied. As shown in Fig. 20 the addition of from 1.0 to 4.0% ethanol by weight has a very markedly inhibitory effect, as judged by the percentage of total products formed. Interestingly enough 0.5% ethanol appears to have a slight stimulating effect on the strain used. The reason for this effect has not been determined.

A few preliminary experiments that were carried out on the periodic removal of ethanol from mashes by distillation were not promising. After distillation of the mash, resterilization, and reinoculation, fermentation proceeded very

slowly. It seems likely that the sugar decomposition incident to the second sterilization had an adverse effect on the organism.

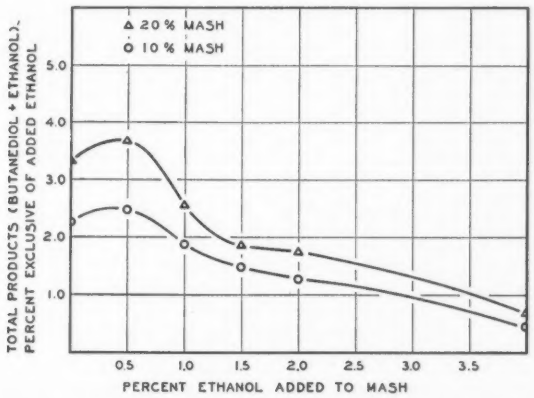


FIG. 20. *The effect of added ethanol on yield of 2,3-butanediol and ethanol on 10 and 20% whole wheat mashes, three-day fermentation period.*

Since it would be very difficult to remove the butanediol from partly fermented mashes without serious effects on the unfermented residues no experiments along this line were attempted.

pH Control

Although the pH of an unbuffered wheat mash lies very close to neutral (6.8 to 7.0), the production of organic acids during the course of the fermentation causes the pH to drop rapidly to the region of 5.0 to 5.5. The simplest method of preventing this drop is to add an excess of calcium carbonate to the mash. In order to determine the amount of calcium carbonate that should be added to a wheat mash to give satisfactory yields of 2,3-butanediol and ethanol, an experiment was designed in which 0, 0.25, 0.50, 0.75, 1.0, 1.5, and 2.0% calcium carbonate was added to 15% mashes. The organisms used for fermentation were *C*3 (2) and *C*4(2). The fermented mashes were analysed at 72 and 96 hr. The average glycol content of all mashes containing calcium carbonate is 2.70%, while mashes without carbonate have a glycol content of 2.23%. The corresponding ethanol values are 1.42 and 1.01. As shown in Table VIII the carbonate level is without significance when averaged over the whole experiment. However, when these levels are examined in greater detail, it is apparent that 2% carbonate differs significantly from the others in that a differential response with different organisms is indicated. The significance of the interaction is accounted for by the fact that organism *C*4(2) shows a decrease at carbonate levels above 1.5%. The most important observation is that the carbonate content of the mash can be reduced to 0.25% without adversely affecting the yield of butanediol and ethanol. Approximately 60 strains were tested on mashes containing no

TABLE VIII

ANALYSIS OF VARIANCE OF 2,3-BUTANEDIOL AND ETHANOL PRODUCTION BY FERMENTATION OF WHEAT MASHES CONTAINING DIFFERENT AMOUNTS OF CALCIUM CARBONATE

Source of variance	Degrees of freedom	Mean square	
		Butanediol	Ethanol
Carbonate	6		
No carbonate vs. carbonate	1	0.7574**	0.5929**
Levels of carbonate	5	0.0164	0.0066
Carbonate × time	6	0.0087	0.0017
Carbonate × organisms	6		
2% vs. other × organism	1	0.1982**	0.0613*
Other levels × organism	5	0.0104	0.0089

* *Exceeds mean square error 5% level of significance.*
** *Exceeds mean square error 1% level of significance.*

carbonate and 1% carbonate. With one or two exceptions lack of carbonate was definitely inhibitory. The pH fell to between 5.0 and 5.5 whereas in the carbonate-treated mashes it rarely went lower than pH 6.0.

Experiments were also carried out on the effects of different carbonates. Results with calcium carbonate, magnesium carbonate, and barium carbonate are shown in Table IX. As judged by total products formed the latter two compounds are not as favourable as calcium carbonate at equivalent per-

TABLE IX

EFFECT OF DIFFERENT CARBONATES ON THE FERMENTATION OF A 15% MASH BY *N.R.R.L. 510*, THREE DAYS AT 30° C.

Carbonate, %	Ethanol, %	Glycol, %	Total products, %	Ratio
0.5 $CaCO_3$	0.70	1.21	1.91	1.73
2.0 $CaCO_3$	0.76	1.22	1.98	1.60
0.25 $BaCO_3$	0.65	1.13	1.78	1.74
0.5 $BaCO_3$	0.62	1.03	1.65	1.66
1.0 $BaCO_3$	0.56	0.98	1.54	1.77
2.0 $BaCO_3$	0.74	1.05	1.79	1.42
0.25 $MgCO_3$	0.69	1.00	1.69	1.45
0.5 $MgCO_3$	0.84	1.07	1.91	1.15
1.0 $MgCO_3$	0.64	0.78	1.42	1.22
2.0 $MgCO_3$	0.70	0.70	1.40	1.00

centages. A rather striking effect is the reduction of the diol–ethanol ratio brought about by magnesium carbonate. The total products varied very little whereas with increasing proportions of magnesium carbonate the ratio dropped steadily, ultimately, with 1.0% magnesium carbonate, reaching a value of less than one.

The addition of excess carbonate to wheat mashes has one undesirable feature in that it increases the ash content of the feed residues and may reduce its value. For this reason it was thought desirable to carry out a number of experiments using ammonia to adjust the pH. So far only a few experiments have been made but they indicated that normal fermentations with equivalent yields of diol and ethanol could be obtained. The best results were obtained in the 6.0 to 6.5 pH range. At higher and lower pH values the yield of total products was reduced. At a pH below 6.0 the diol/ethanol ratio was higher than in pH ranges nearer neutral.

One of the chief difficulties in using ammonia is to obtain accurate pH control. So far the pH has been adjusted through intermittent additions whenever the pH indicator added to the mash showed change in colour. It is recognized that the electrometric rather than colorimetric control would be more satisfactory, hence further work on this problem is now being carried out.

Nutritional Studies

For the development of an economical industrial process on the production of 2,3-butanediol from wheat it may be preferable to process the grain and remove the non-fermentable fractions prior to fermentation. Before this can be done, however, a more comprehensive knowledge of the nutritional requirements of *Aerobacillus polymyxa* is essential. Since satisfactory fermentations cannot be obtained on pure starch media with the usual inorganic supplements an attempt has been made to assess the role played by the different wheat fractions such as bran, gluten, and water-soluble constituents in the fermentation. The problem has been studied in several different ways; first, by the addition of extra starch to whole wheat flours in order to determine whether these factors were present in excess of normal requirements; second, by separation of the various wheat fractions and their subsequent recombination with the starch; third, fortification of the various mashes with yeast extract or other suitable supplements.

The effects of adding purified wheat starch to a whole wheat mash are shown in Table X. Starting with a 10% whole wheat mash, 1.0, 2.0, 5.0,

TABLE X

EFFECT OF ADDED STARCH ON FERMENTATION* OF WHOLE WHEAT MASHES

——	% 2,3-Butanediol + ethanol	Increase over whole wheat mash	Theoretical increase if added starch completely used
10% whole wheat mash	2.29	—	—
10% whole wheat mash 1.4% starch	2.51	0.22	0.55
10% whole wheat mash 2.0% starch	2.90	0.61	1.10
10% whole wheat mash 5.0% starch	3.25	0.96	2.75
10% whole wheat mash 10% starch	3.74	1.45	5.50

**Fermentation 72 hr. at 30° C. using culture N.R.R.L. 510.*

and 10.0% of starch (based on total weight of the mash) was added and a 72-hr. fermentation was then carried out. The ratio of starch to the other constituents of wheat was proportionately increased until it was about three times greater than in the whole wheat control. As shown in the table the additional starch was very inefficiently utilized, thus showing that the nutritive properties of certain non-starchy constituents of wheat cannot be diluted without impairing the fermentation. In fact it can readily be demonstrated that whole wheat mash itself is not an ideal medium for this organism, since as shown in Table XI, the addition of yeast extract enhances the fermentation.

TABLE XI

EFFECT OF WHEAT FRACTIONS AND YEAST EXTRACT ON FERMENTATION

Wheat fraction	2,3-Butanediol + ethanol, %			
	Strain 233*b*(2)		Strain 510*R*	
	Yeast extract added			
	None	0.25%	None	0.25%
Whole wheat flour	3.33	3.56	3.17	3.54
Starch + wash water	2.49	3.00	2.56	3.03
Starch + wash water + bran	2.90	3.26	3.00	3.30
Whole wheat flour	2.78	3.29	3.12	3.26
Starch + wash water + bran	2.68	3.00	2.93	3.09
Starch + wash water + bran + half the gluten	2.71	2.90	2.82	3.00
Starch + wash water + bran + all the gluten	2.78	3.01	2.80	2.99

The beneficial effect of yeast extract on the whole wheat mashes, as shown in Fig. 21 is most marked when it is added to the extent of 0.25 to 0.50%, whereas increases of over 1.0% may depress the rate of fermentation. Using mashes prepared from patent flour, a greater percentage of yeast extract is beneficial. Substantially the same stimulatory effects may be obtained by the addition of corn steep liquor in concentrations of the order of 3.0%.

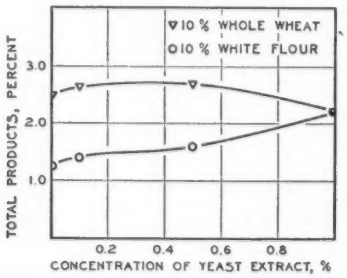


FIG. 21. *Effect of yeast extract on fermentation of whole wheat and white flour.*

The studies on the effects of the different wheat fractions on the fermentation have been carried out in two different ways. For most of the work the various constituents of the whole wheat flour were obtained by washing out and separating by screening the starch, bran, and gluten. The starch was never separated from the wash waters, which contain nutritive constituents. Various recombinations of the fractions were made as indicated in Table XI. The first experiment dealt with all the constituents except the gluten. It is apparent that starch + wash water is not a satisfactory fermentation medium, but is enhanced considerably by the addition of the bran. Even the most favourable mash never equalled one of whole wheat flour, but this can be explained on the basis of a slightly lower starch content of the reconstituted mash. The addition of yeast extract was markedly beneficial. The addition of gluten to all the other constituents did not have any beneficial effects. These experiments indicate that the nitrogen nutrition of *Aerobacillus polymyxa* is largely met by the soluble nitrogenous fraction rather than by the gluten.

In the foregoing experiment the effect of bran was presented as a composite including the effects of bran, shorts, and germ (Table XI). It was thought that the relative importance of these different constituents might be assessed by adding different percentages of commercial samples of each to pure starch and then carrying out fermentations on the cooked mashes. These results are shown in Table XII. Addition of less than 5% (on weight of mash) of any of these fractions was not very effective, but moderately good fermentations were obtained when 7.5 or 10.0% were added. This type of medium does not hold much promise of being of practical value, since the amounts of

TABLE XII

FERMENTATION OF 7.5% STARCH SOLUTIONS BY *N.R.R.L.* 510 IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF BRAN, SHORTS, AND WHEAT GERM, THREE DAYS AT 30° C.

Material	Percentage	Ethanol, %	Glycol, %	Total products, %	Theoretical, %
Wheat germ	1.0	0.31	0.49	0.80	19
Wheat germ	2.0	0.53	0.63	1.16	28
Wheat germ	5.0	1.14	1.84	2.98	72
Wheat germ	7.5	1.28	2.16	3.44	83
Wheat germ	10.0	1.35	2.19	3.54	86
Shorts	1.0	0.11	0.31	0.42	10
Shorts	2.0	0.48	0.73	1.21	29
Shorts	5.0	1.37	1.85	3.22	78
Shorts	7.5	1.28	1.92	3.20	78
Shorts	10.0	1.31	2.27	3.58	87
Bran	1.0	0.11	0.43	0.54	13
Bran	2.0	0.21	0.50	0.71	17
Bran	5.0	1.20	1.70	2.90	70
Bran	7.5	1.19	1.85	3.04	74
Bran	10 0	1.27	2.14	3.41	83

germ, shorts, and bran required to give satisfactory fermentations are in excess of the amounts normally present in wheat. Although the above medium prepared with purified starch does not give good results, the addition to it of the wash waters obtained by the dough washing method of separation of the constituents holds more promise. This latter approach to the problem is therefore being studied in greater detail.

Environmental Factors and Rate of Fermentation

As a result of the foregoing studies on factors that affect the fermentation a gradual improvement in the yield of 2,3-butanediol and ethanol was achieved. This increase gradually approached a maximum beyond which further increases were difficult to obtain. Probably the greatest single contribution to this increased yield was made in the selection of high yielding strains. As an example of the results being obtained, the yields of diol and ethanol by four of the best strains are shown in Fig. 22. The results are typical of those

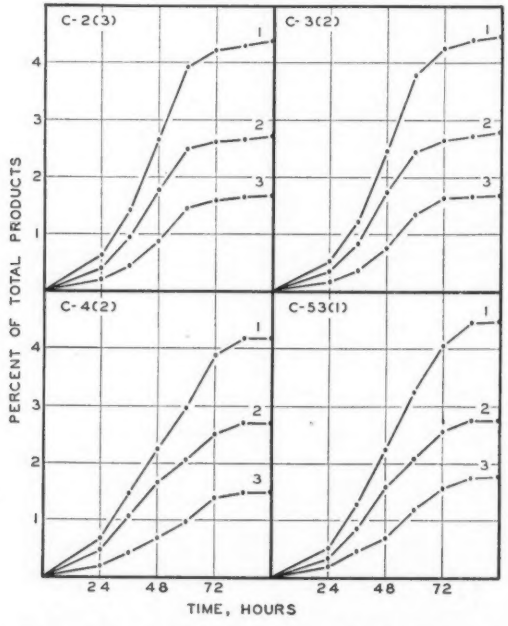


FIG. 22. *Rate of fermentation of 15% whole wheat mashes by four strains of Aerobacillus polymyxa as shown by production at 12-hr. intervals of (1) total products, (2) 2,3-butanediol, (3) ethanol.*

obtained when 300 cc. of 15% whole wheat mash in 500 cc. Erlenmeyer flasks was used. Two strains, *C*2 (3) and *C*3(2) had produced slightly over 4% total products in 72 hr. and judging from the very slight increase that resulted at 96 hr. the fermentation was almost complete. *C*53(1) eventually

produced the highest yield but there was a 5% increase after 72 hr. The diol–ethanol ratio for most of the strains was approximately 1.7 : 1.0.

Preliminary experimentation indicated that a temperature of about 30° C. was an optimum for the growth of the *Aerobacillus* organisms. Effects of fermentation temperature and time were determined by the following experiment. Four organisms (*C*38(1), 510*R*, *S*13, and *C*42(3)) were grown in duplicate at temperatures of 30°, 32.5°, 35°, and 37° C. and the mashes analysed at 48 and 72 hr. The analytical results were subjected to the analysis of variance procedure (Table XIII).

TABLE XIII

ANALYSES OF VARIANCE OF 2,3-BUTANEDIOL AND ETHANOL PRODUCTION BY FERMENTATION AT DIFFERENT TEMPERATURES AND TIMES

Source of variance	Degrees of freedom	Mean square	
		Butanediol	Ethanol
Temperature	3		
30° vs. other temperatures	1	0.9606**	0.8138**
Others	2	0.0596	0.0102
Temperature × organism	9		
30° vs. others × organism	3	0.1006**	0.0522**
Other temperature × organisms	6	0.0147	0.0105
Temperature × time	3		
30° vs. others × time	1	0.3726**	0.1420**
Other temperatures × time	2	0.0682*	0.0670**
Temperature × organism × time	9	0.0121**	0.0047**
Duplicates	32	0.0030	0.0014

* *Exceeds mean square error 5% level of significance.*
** *Exceeds mean square error 1% level of significance.*

It is evident from Tables XIV and XV that 30° C. gives, in general, a significantly lower yield of 2,3-butanediol and ethanol than the other temperatures. At 48 hr. fermentation time all organisms showed inferior yields at 30° C. although *S*13 did relatively better than the others. At 72 hr. the yield for *C*38(1), 510*R*, and *C*42(3) was still inferior at 30° C. while *S*13 gave its best yield at this temperature. In general at 72 hr. the yield tends to fall off at the higher temperatures.

Practical considerations may necessitate the use of different fermentation times and organisms since the latter are subject to variation. Under these conditions the differential effects of time and organism cannot be stated with assurance. In general, it appears that a fermentation temperature of about 32.5° C. would be the most satisfactory.

TABLE XIV

INTERACTION EFFECTS OF ORGANISM AND FERMENTATION TEMPERATURE ON 2,3-BUTANEDIOL AND ETHANOL YIELD

Organism	Butanediol, %		Ethanol, %	
	Temperature		Temperature	
	30° C.	Mean of 32.5° to 37° C.	30° C.	Mean of 32.5° to 37° C.
*C*38(1)	1.60	1.94	0.86	1.13
510*R*	1.64	2.02	0.82	1.10
*S*13	1.92	1.93	1.00	1.09
*C*42(3)	1.77	2.18	0.98	1.38
Mean	1.73	2.02	0.92	1.18

TABLE XV

INTERACTION EFFECTS OF FERMENTATION TIMES AND TEMPERATURES ON 2,3-BUTANEDIOL AND ETHANOL YIELD

Fermentation temperature, ° C.	Butanediol, %			Ethanol, %		
	Time, hr.			Time, hr.		
	48	72	Average	48	72	Average
30	1.35	2.12	1.73	0.66	1.16	0.92
32.5	1.77	2.34	2.05	0.98	1.40	1.19
35.0	1.88	2.23	2.05	1.08	1.30	1.19
37.0	1.78	2.11	1.95	1.05	1.24	1.15

Aerobic and Anaerobic Fermentations

From a theoretical standpoint it might be reasonably anticipated that diol and ethanol, being reduced compounds, would be more readily formed under anaerobic conditions. Some preliminary experiments carried out under anaerobic conditions produced by passing nitrogen and hydrogen through wheat mashes has markedly increased the rate of fermentation. Under these conditions the time of fermentation was reduced from 72 to 48 hr., and the diol–ethanol ratio decreased from 2 : 1 to approximately 1.3 : 1. Similar treatment with oxygen causes a marked inhibition of ethanol formation and a slight reduction in the production of diol. The use of air gives a similar but less noticeable effect. This effect is fundamentally a change in rate, and in spite of change in diol–ethanol ratio there is no diminution in the total products formed provided the fermentation period is increased by approximately 50%.

Agitation of the medium with the surface in contact with air produces an effect similar to, but less marked than, that produced by aeration. Lack of

uniformity in ratios and in rate of fermentation in earlier work is now partially explainable as being due to irregular shaking of flasks during the fermentation period. Another important factor that influences both rate of fermentation and diol–ethanol ratio is the volume–surface relationship of the medium. As pointed out by Katznelson (4) in similar studies, fermentations may be carried to completion more rapidly by using thin layers of mash. A further detailed investigation of this observation has shown it to be related directly to the escape of carbon dioxide from the medium. Rapid removal of the carbon dioxide from fermenting cultures by reduced pressure has greatly increased the rate of the fermentation.

These unexpected and pronounced effects of carbon dioxide and oxygen on the course of fermentation have led to continued studies, which will be reported in due course.

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PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

II. STRAINS OF *AEROBACILLUS POLYMYXA* IN RELATION TO FILTERABILITY AND BUTANEDIOL PRODUCTION[1]

By R. Y. Stanier[2], G. A. Adams[2], and G. A. Ledingham[2]

Abstract

Under laboratory conditions it has been found that most strains of *Aerobacillus polymyxa* split off variants that differ substantially in: colony formation; appearance of wheat mashes after fermentation; filterability of these mashes; and their ability to produce 2,3-butanediol and ethanol. It has been impossible to demonstrate a close correlation between particular colony types and their usefulness for commercial 2,3-butanediol production. By selection it has been possible to obtain strains that are excellent both from the standpoint of mash filterability and product yield. Preservation of cultures in lyophile tubes prevents further variation during storage.

Introduction

Aerobacillus polymyxa is an extremely variable organism. As was mentioned in the introductory paper of this series (1), marked differences in colony appearance between strains can be seen even on first isolation; furthermore, these strains, originally 'pure', will often split up into a number of variant types (judged by colony form) when maintained under laboratory conditions.

At the same time, changes have occasionally been noticed in the power of certain strains to produce butanediol from wheat mashes when tested at intervals of several months; not infrequently a strain originally very active as a butanediol producer will undergo an apparent diminution of fermentative abilities in the course of time. This observation naturally suggested the hypothesis that the variations in colony form that we had observed might be connected with the changes in fermentative ability.

Over the course of a year, colony variants had been isolated from some 20 of our *Aerobacillus* strains; in most cases only two or three variants were obtained from a single strain, but a few strains produced as many as six. In order to test the above hypothesis, an exhaustive comparative study was made of the fermentative properties of these variants, grown on 10% and 15% whole wheat mashes. At the same time the filterability of the fermented mashes was investigated, since this is an important factor from the practical standpoint and preliminary observations had indicated that quite marked strain differences existed with respect to it.

Methods

The methods of studying butanediol and ethanol production from wheat mashes have been outlined in a previous paper of this series (1).

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[2] *Bacteriologist, Biochemist, and Mycologist respectively, Industrial Utilization Investigations.*

The filterability of the fermented mashes was compared by determining the volume of filtrate obtained from a given volume of mash at various intervals of time. The mashes were well shaken and poured into pyrex funnels of 10 cm. diameter containing folded No. 1 Whatman 18.5 cm. filter papers. The filter paper was always filled to capacity; slight differences in mash volume had little or no effect on the subsequent rate of filtration. The amount of filtrate was measured at 25 min. Although this method has a coefficient of variation of about 10% over all the experiments reported here, it was regarded as sufficiently precise for the purpose for which it was used.

All determinations of filterability and butanediol and ethanol production are averages of duplicates.

Experimental Results

Colony Types in Aerobacillus polymyxa

The extent of colony variation that one encounters in *Aerobacillus polymyxa* is so great that it is not possible to present any precise classification of types. Independent variation of several different characters occurs: size, colour (yellow, chalk-white, greyish-white), shape (convex, flat, umbonate, etc.), translucency, consistency, surface. As a result, a very large number of distinct colony types are found, and any attempt at classification, of necessity based on one or two characters, fails to take care of the many exceptions. Plate I shows a few of the diverse types encountered and gives a good idea of the range of variation obtainable. Since we have not been able to discern any correlations between the important practical characteristics of filterability and fermentability on the one hand, and colony form on the other, a further description of the colony variants is not given. This phase of the problem is chiefly of academic interest except in so far as colony variation in a strain may serve to indicate the production of variants possibly differing in fermentative ability.

Mash Type After Fermentation

Depending on the distribution of the solids after fermentation, three characteristic and distinct types of fermented mash may be distinguished (Fig. 11). In Type A, the residual solids (mostly bran and gluten) collect in a spongy mass at the surface, held together by slime and buoyed up by the entrapped gases. The liquid below is clear, and only very rarely is a deposit of solids found at the bottom of the container. In Type B, the solids collect as a loose sediment, with a layer of clear liquid above. In Type *C*, there is no separation, the solids being held in even suspension by copious loose slime formation. Type *C* mashes are often of the consistency of mayonnaise. These differences are shown most clearly when a 10% wheat mash is used, but they are also evident in 15% mashes, although generally much less clear-cut.

The type of mash produced by a particular strain of *Aerobacillus polymyxa* is generally constant, and might serve as a useful preliminary indication of the practicability of the strain for commercial butanediol production. As shown in Table I, Type *C* mashes are characterized by very low filterability,

TABLE I

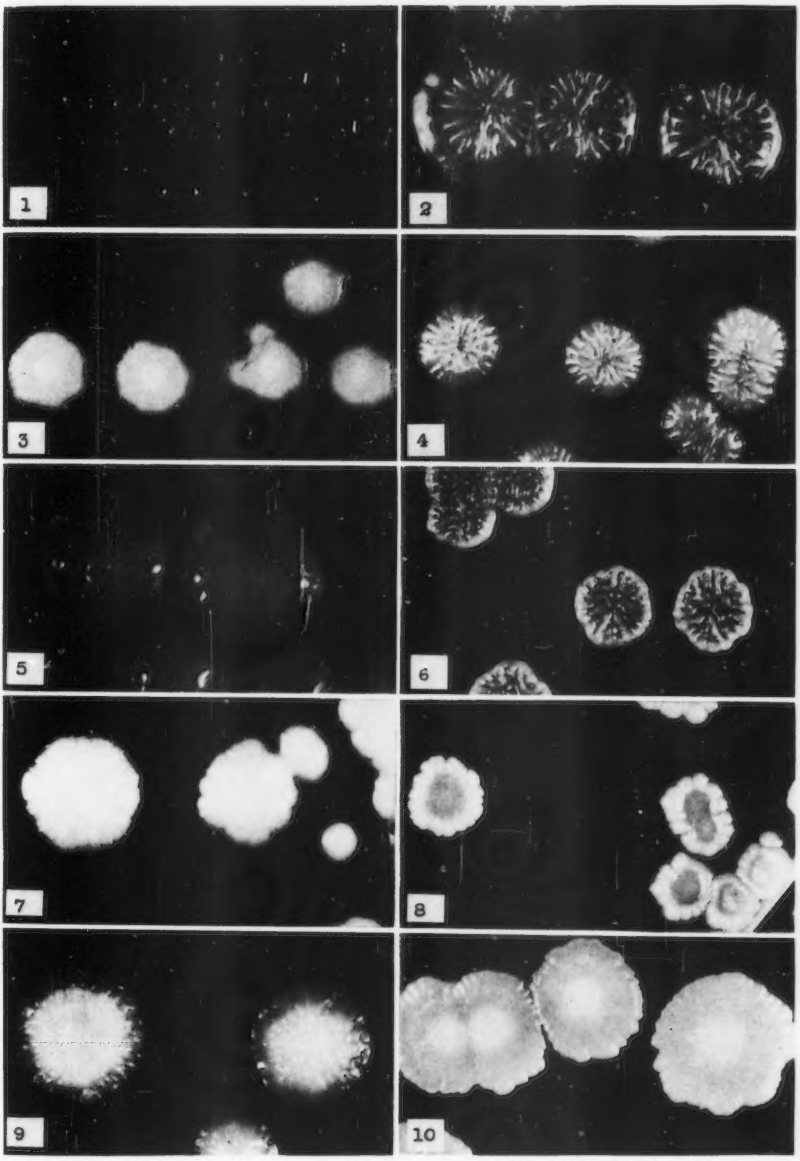
COMPARATIVE YIELDS, TYPE OF MASH, AND FILTERABILITY OF *Aerobacillus* VARIANTS ON 10% WHOLE WHEAT MASH, FERMENTED THREE DAYS

Yield of 2,3-butanediol + ethanol, %	Volume of filtrate, ml.							
	Over 60		60 to 40		40 to 20		Under 20	
	Culture No.	Type of mash	Culture No.	Type of mash	Culture No.	Type of mash	Culture No.	Type of mash
Over 2.5			*C*2(3) *C*53(1)	*B* *A*	*UA*233*b*(3)	*A*		
2.5 to 2.0	*C*3(1)	*B*	*C*3(3) *C*3(5) *C*23(1) *UA*233*b*(2) *S*13(2) *C*38(1)	*B* *B* *B* *A* *B* *A*	*C*47(3) *C*2(1) *C*2(2) *C*3(2) *C*6(3) *C*23(2) *UA*233*a*(1) *UA*233*a*(2) *UA*233*a*(4) *C*4(2) *C*5(3) *C*38(2) *C*47(1)	*B* *A* *B* *B* *B* *B* *A* *A* *B* *B* *A* *A* *B*	*C*3(6) *UA*233*a*(3) *C*23(3) *UA*233*a*(5) *S*13(1) *C*4(3) *C*8(1) *C*56(3)	*C* *C* *B* *C* *C* *A* *C* *A*
2.0 to 1.5			*C*3(4) *C*6(4) *C*6(2) *C*8(4) *C*56(1)	*B* *B* *A* *A* *B*	*C*53(4) *C*5(2) *C*8(3) *C*20(2) *C*56(2)	*A* *A* *A* *B* *A*	*C*6(1) *C*4(1) *C*12(1) *C*38(3)	*B* *C* *C* *C*
Under 1.5			*C*20(4)	*B*	*C*53(3) *C*12(2) *C*20(1)	*B* *B* *B*	*C*53(2) *C*5(1) *C*20(3)	*A* *C* *B*

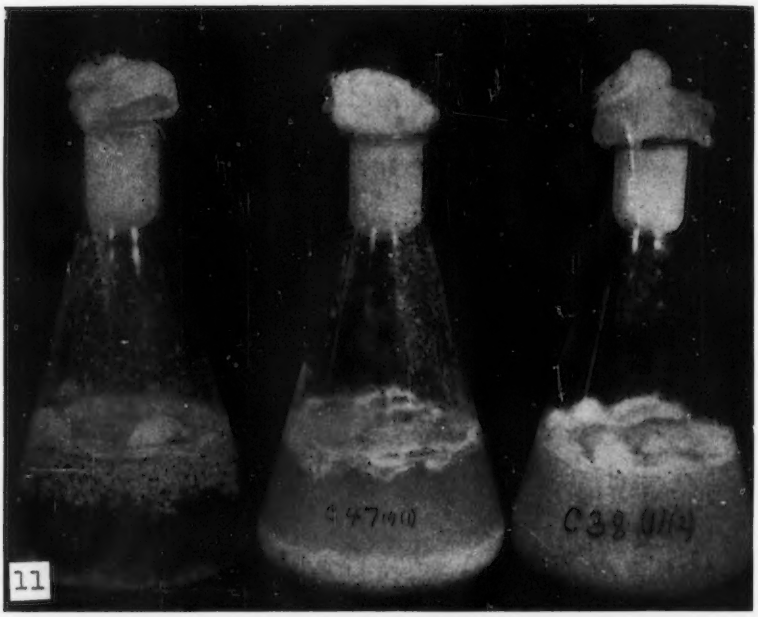
and even though an occasional strain may be a high butanediol producer, it would be of little practical value from the industrial standpoint. Between Type *A* and Type *B* mashes, there is little to choose either on the basis of filterability or product yield. Either type of mash may be formed by high or low butanediol-producing strains.

Comparison of Variants for Filterability and Yield of 2,3-Butanediol and Ethanol

Tables I and II present data on the filterability and yield of total products from 10 and 15% whole wheat mashes respectively. It was hoped that filterability might provide some indication as to the probable product yield since this would allow for rapid assessment of new strains and variants. However determination of the degree of correlation between filterability and product yield over all organisms in Table I gave a coefficient of .18 for 10% mashes. The corresponding coefficient shown in Table II was .43, which was significant. However this is not of sufficient magnitude to justify its use for prediction purposes. It was further found that the correlation coefficients between 10 and 15% mashes with respect to filterability and total products were .68



Some common colony types of Aerobacillus polymyxa. All photographs are strictly comparable with respect to age, media, and magnification. FIG. 1, *C23(2)*; FIG. 2, *C38(1)*; FIG. 3, *C2a(1)*; FIG. 4, *C53(4)*; FIG. 5, *UA233b(5)*; FIG. 6, *C5(1)*; FIG. 7, *C5(2)*; FIG. 8, *C2(3)*; FIG. 9, *C42(3)*; FIG. 10, *C1*.



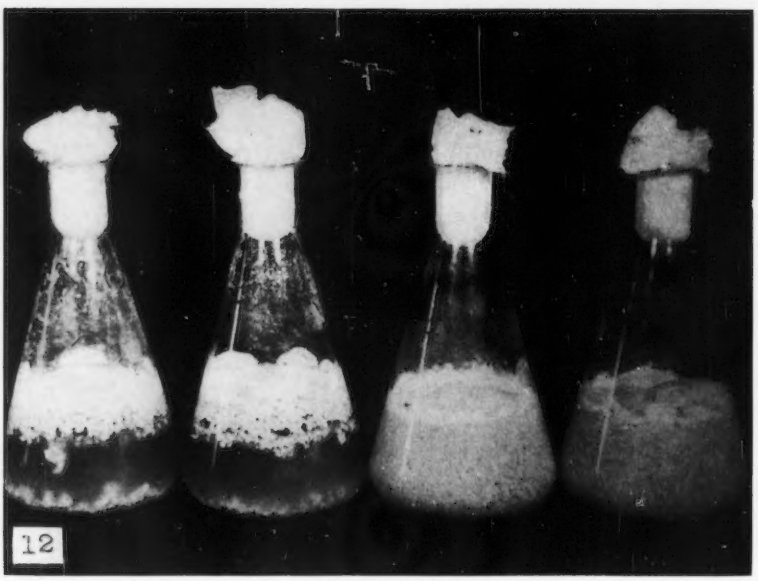


FIG. 11. *The three types of fermented mash produced by Aerobacillus polymyxa; Type A (left), Type B (middle), and Type C (right).* FIG. 12. *Duplicate flasks of C38 (2) (left) and C38(3) (right), variants of a single strain that produce different types of fermented mashes. All flasks contain 10% whole wheat mashes fermented three days at 30° C.*

TABLE II

COMPARATIVE YIELDS AND FILTERABILITY OF *Aerobacillus* VARIANTS ON 15% WHOLE WHEAT MASH, FERMENTED THREE DAYS

Yield of 2,3-butanediol + ethanol, %	Volume of filtrate, ml.			
	Over 30	30 to 20	20 to 10	Below 10
Over 3.5	*C*53(1)	*C*2(3) *C*3(2) *C*3(3) *C*4(2) *C*5(3) *C*20(4) *C*38(2) *UA*233*a*(4)	*C*3(6) *C*14(1) *C*20(3) *UA*233*a*(1) *UA*233*b*(3)	
3.5 to 3.0	*C*3(1) *C*6(2) *S*13(2) *UA*233*b*(1) *UA*233*b*(2)	*C*3(5) *C*8(3) *C*8(4) *C*19(1) *C*19(2) *C*38(1) *C*47(1) *C*47(2)	*C*2(1) *C*23(3) *UA*233*a*(3)	*C*6(5) *C*20(2) *S*13(1)
3.0 to 2.5		*C*2(2) *C*6(3) *C*6(4) *C*56(1)	*C*5(2) *C*14(2) *C*23(2) *C*42(3) *C*53(2) *C*56(3)	*C*4(3) *C*8(2) *C*8(4) *C*42(1) *C*53(4) *UA*233*a*(5)
2.5 to 2.0		*C*23(1)	*C*6(1) *C*38(3) *C*42(2) *C*42(4)	*C*4(1) *C*53(3)
Below 2.0			*C*3(4)	*C*5(1) *C*20(1)

and .65 respectively. It follows therefore that organisms that produce readily filterable mashes and high yields of products on 10% mashes will tend to give similar results on 15% mashes.

Further examination of the data presented in Tables I and II show clearly that very large differences occur in both filterability and product yield even between the variants from a single original strain. In some cases a very active variant has arisen from a strain that was originally a mediocre fermenter (1). An excellent example of this is *C*53. In these experiments four variants of this original strain were tested. *C*53(1) is an outstanding variant, being high in filterability and product yield. The other three variants, *C*53(2), C53(3), and *C*53(4) are all definitely inferior. Another example is *C*3, which produced six variants, five of which were much superior and one inferior in yield to the original parent. It should be noted, however, that improvement in productivity was not uniformly accompanied by good filterability. Other

strains have regressed with respect to yield of total products, as demonstrated by *C*42. The original culture was a moderately high diol producer but the four variants to which it has given rise are inferior to it. In general it is impossible to predict by any means other than actual test whether new variants from any particular strain will have desirable fermentative charteristics.

Further Selection for Maximum Yield

On the basis of the previous comparative tests on 10 and 15% mashes, six strains that were outstanding for their yield of total products and at the same time produced mashes that filtered with reasonable rapidity, were selected for further study and comparison. The yields of butanediol and ethanol and the filterability of these strains grown on 15% mashes were studied at two, three, and four days. The results are presented in Figs. 13 and 14. It will be seen that with the exception of *UA*233*b*(3) there is little to choose between the six as far as the yields of butanediol and ethanol production are concerned. On the basis of filterability, *C*2(3) is definitely the best strain at three days, although it is not as good as *C*4(2) and *C*53(1) at four days.

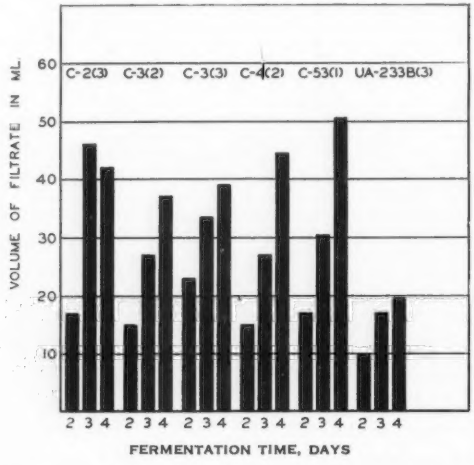


FIG. 13. *Filterability (25 min.) of 15% whole wheat mashes fermented two, three, and four days at 30° C. by six of the best selected variants.*

Further experiments on the yields of butanediol and ethanol by *C*2(3), *C*3(2), *C*3(3), *C*4(2), and *C*53(1) have shown that the slight differences between them obtained in the experiment presented above are not consistent, and for practical purposes they may be regarded as equally good fermenters. For a three day fermentation, the butanediol production ranges from 2.4 to 2.8% and the total products from 3.8 to 4.3%, while for a four day fermentation

the figures are 2.3 to 2.9% and 4.2 to 4.5% respectively. It is questionable whether the slight increase in yield at four days is sufficient compensation for the extra time involved, although the desirability of obtaining a complete fermentation may make it worth while.

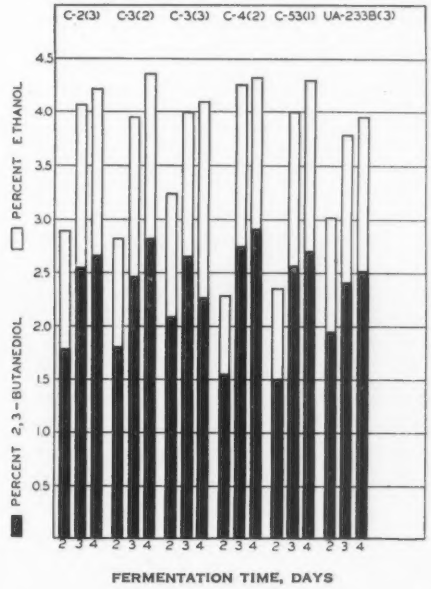


FIG. 14. *Yields of 2,3-butanediol and ethanol by six of the best selected variants grown on 15% whole wheat mashes at 30° C., for two, three, and four days.*

It is evident that with these extremely active strains, a very close approximation to the theoretically possible yield of total products can be obtained. For a 15% wheat mash containing 55% starch, the theoretical is 4.53% total products, so that at four days these strains produce 90 to 100% of the possible total products.

Maintenance of Active Cultures Under Plant Conditions

The work reported in this paper shows the paramount importance of working with the right strains for a successful butanediol fermentation by *Aerobacillus polymyxa*. Furthermore, continuous precautions must be taken in order to avoid losses due to the 'degeneration' of a good strain through the development of variants with inferior fermentative abilities or filtering properties. Probably if butanediol production by *Aerobacillus polymyxa* becomes a large scale industrial operation, it will be necessary to carry on continued bacteriological investigations on the strains and variants.

In order to ensure the availability of vigorous cultures for pilot plant operations, the best strains are being preserved by drying. It is a simple matter to dry 100 ampoules of a culture, and this treatment has the great advantage of stabilizing the organism and preventing further variation during storage. These dried cultures will be used as the 'starters' for inoculum in the pilot plant; on the basis of two runs a week, 100 such ampoules will thus supply sufficient material for almost one year's operation.

References

1. LEDINGHAM, G. A., ADAMS, G. A., AND STANIER, R. Y. Production and properties of 2,3-butanediol. I. Fermentation of wheat mashes by *Aerobacillus polymyxa*. Can. J. Research, F, 23 : 48-71. 1945.

PRODUCTION AND PROPERTIES OF 2,3-BUTANEDIOL

V. SMALL-SCALE PRODUCTION UNIT[1]

By Dyson Rose[2] and W. S. King[2]

Abstract

A small-scale process for producing 2,3-butanediol by fermentation of approximately 25 gal. of whole wheat mash per week is described. Although simple and unspecialized, the equipment proved satisfactory for the purpose of providing fermentation and recovery data and sufficient diol for experimental purposes. Data are given which show that with careful control of the operating conditions the fermentations obtained were entirely satisfactory but that losses in the recovery process lower the over-all recovery of diol to about 55% of that shown by analysis of the fermented mashes.

Introduction

During the course of extensive studies of the production and utilization of 2,3-butanediol in the National Research Laboratories (1) a small experimental unit was designed in order to obtain data on the production and recovery, as well as to supply diol for chemical studies. The apparatus developed has proved quite successful and since it is applicable to a wide range of uses the design and method of operation may prove of value to other laboratories. For this reason they are presented in some detail together with certain experimental data that serve to indicate the results obtainable.

Equipment

A general view of the equipment is shown in Fig. 3, while Fig. 1, the operational flow diagram, outlines the equipment and indicates the general process. As available materials were used wherever possible some of the simpler units were not especially designed for their purpose. These parts are not described in detail.

1. *Cooking and Mashing Equipment*

Details of the design of this vessel are shown in Fig. 2. It is a jacketed vessel of 10 gal. capacity with a maximum operating pressure of 40 p.s.i.g. The specially designed stirring mechanism enters through the bottom of the vessel so that it may be used while the vessel is open. This type of stirrer has the added advantage that it leaves the lid of the vessel free from weighty gears and glands. The weight of the stirrer is taken by the thrust type bearing located on top of the shaft housing while the two packing glands prevent leakage of mash past the shaft. Lubrication is obtained by packing the shaft and glands with a graphite grease mixture every 40 to 50 days.

[1] *Manuscript received September 9, 1944.*

Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as Paper No. 18 on the Industrial Utilization of Wastes and Surpluses, and as N.R.C. No. 1240.

[2] *Biochemist, Industrial Utilization Investigations.*

It is essential that the plumbing of this vessel, including the mash line, be arranged as shown in Fig. 2, so that live steam may be admitted intermittently

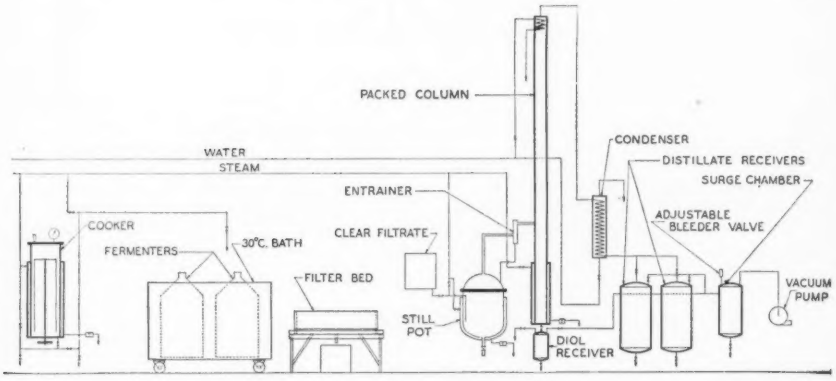


FIG. 1. *Operational flow diagram.*

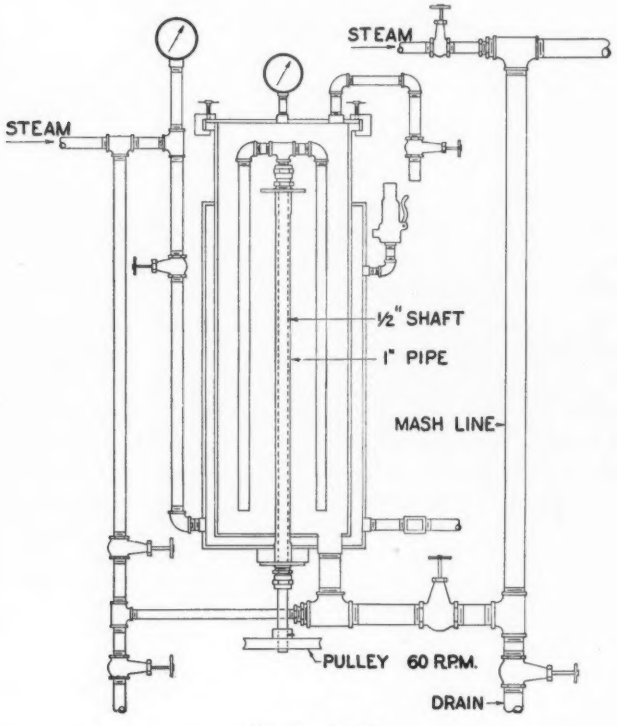


FIG. 2. *Cooker.*

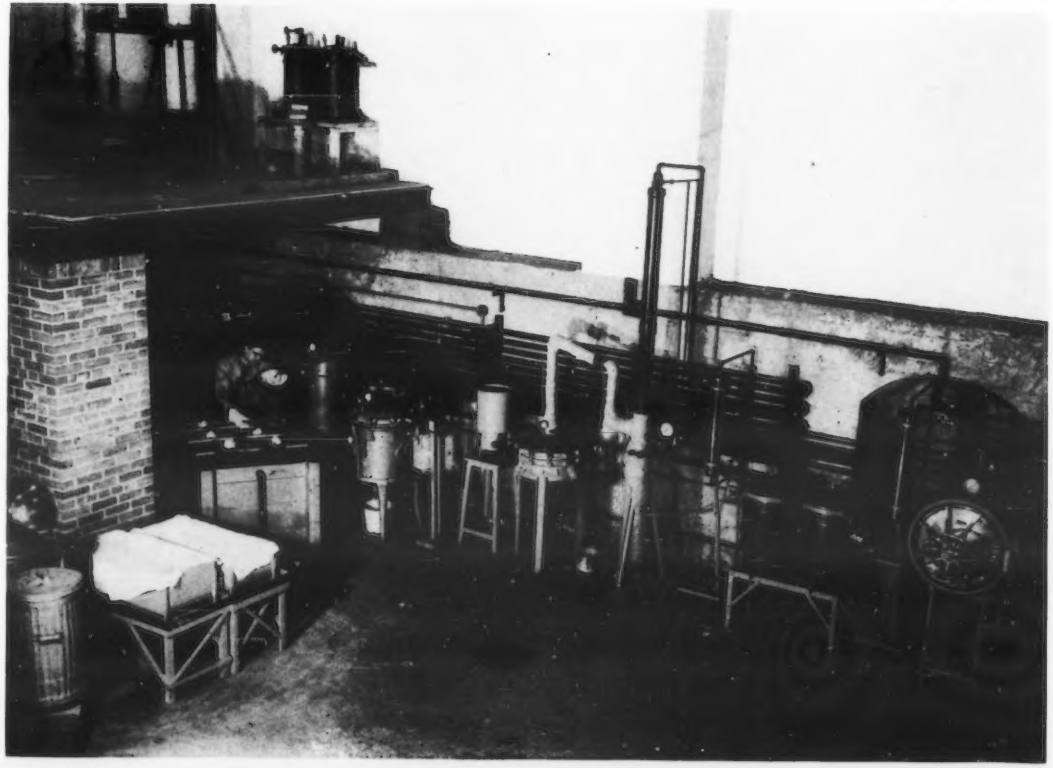


FIG. 3. *General view of the equipment.*

through the valve close to the cooker. Heat conductivity alone does not ensure sterility at this point.

2. *Fermenting and Filtering Equipment*

Since the fermenters are autoclaved individually they are of extremely simple construction. Almost any metal drum of about 5 gal. capacity and provided with a suitable outlet and cotton plug may be used. When intermittent agitation of the mash is required it can be simply arranged for by providing the fermenter with a threaded outlet and screw-on cap. The cap is sterilized separately and replaces the cotton plug only while the vessel is being shaken.

The necessary temperature regulation during the fermentation period is obtained by means of a thermostatically controlled water-bath.

The filtration of the protein–bran residues from fermented grain mashes presents a difficult problem, but for laboratory purposes where speed is not essential a simple type of filter bed has been found adequate. Cheesecloth suspended over a wood and woven-wire frame gives, with the exception of the first few litres, which are refiltered, a beer sufficiently clear for distillation. Two frames providing $4\frac{1}{2}$ sq. ft. of filtering surface each handled most mashes with sufficient rapidity to ensure an adequate supply of filtrate for daily operation of the distillation unit.

3. *Distillation Equipment*

The distillation unit consists of the following pieces of equipment:

(1) The feed reservoir;
(2) The still-pot;
(3) The column, including the re-boiler section;
(4) The diol receiver;
(5) The condenser;
(6) The condensate receivers;
(7) The vacuum pump.

No detailed diagrams are presented for these pieces of apparatus as they are mostly of a simple or standard type of construction. The relative position and interconnection of all are shown in the flow diagram (Fig. 1).

The feed reservoir is an open type vessel of 30 litre capacity from which a line leads through a needle valve into a sparger ring in the base of the still-pot. The feed may be preheated by means of a small steam coil in the vessel while the rate of feed is regulated by means of the valve.

The still-pot is a jacketed vessel of 45 litre capacity designed for operation under full vacuum with 15 p.s.i.g. of steam in the jacket. It is provided with dual sight glasses to enable the operator to watch the rate of feed and also to guard against excessive frothing. In addition, a thermometer is inserted near the top of the pot to record the temperature of the vapours passing to the column. Live steam may be injected into this pot through the same sparger through which the feed enters.

The column consists of 10 ft. of 4 in. pipe packed throughout its entire length with 1 in. Raschig rings. It is of iron construction although copper would be preferable. The feed vapour enters the column at a height of 3 ft. from the base. Reflux is provided by a cooling coil situated in the upper 6 in. of the column but the rate of reflux is obtainable only by calculation from the temperatures and flow of the cooling water. The lower 2 ft. or re-boiler section of the column is provided with a steam jacket capable of withstanding up to 40 p.s.i.g. The diol may be drained off either intermittently or continuously into the diol receiver, which is so designed that it may be closed off from the column, emptied, and evacuated as required. (cf. Fig. 1).

The condenser and condensate receivers are of standard design and are constructed of copper. Two of the latter are provided so that the distillate may be divided into fractions if desired, it being possible to interchange them during operation.

The vacuum is provided by a dry vacuum pump of slightly excess capacity so that it may be regulated by an adjustable bleeder valve that admits air to the surge chamber whenever the vacuum exceeds the desired amount.

The Process

Preparation of the Mash

The mash most extensively used in this work contains between 10 and 11% of dry matter and is made up in the following proportions:

Water, cold	45 litres
Wheat, ground	6000 gm.
Calcium carbonate	400 gm.
Malt	60 gm.

About two-thirds of the water is placed in the cooker and the wheat and carbonate are added slowly with agitation, then the remaining water is used to wash down the upper parts of the vessel so that no dry material remains. The cold, well mixed mash is then heated to 70° C. and held at this temperature for 20 min. after the malt, previously slurried in a small amount of cold water, has been added. Following the malting, the lid of the cooker is clamped in place, with the blow-off valve open, and the mash is heated until all the enclosed air has been driven out. This valve is then closed and the cook is carried out at 10 p.s.i.g. for one hour.

Prior to the malting period and at least once during the cook, live steam is injected into the cooker from the mash lines. These lines are kept under pressure throughout the cook to ensure sterility, the condensate being drained off periodically. Following the cook and as a further precaution against non-sterile particles against the valve the first half-litre of mash is blown out of the drain leading away from the mash line. The main portion of the mash is then blown to the fermenters and cooled in the water bath overnight.

Fermentation and Filtration

The fermenters are cooled to 30° C., inoculated with 2 litres of culture, and fermentation is allowed to proceed for 96 hr. When fermentation is complete the pH of the mash is adjusted to approximately 8 by the addition of slaked lime. It is then allowed to settle for 8 to 12 hr. so that about half of its volume can be decanted as clear beer. The remainder is poured on to the filter beds and filtration is allowed to proceed until no free liquid remains on the bed; then wash water is added. The operation is generally complete in 24 hr.

Distillation

The clear beer obtained by decantation and filtration is neutralized by the addition of sulphuric acid before distillation. During the initial stage of the distillation the vacuum is adjusted to leave an absolute pressure of about 30 cm. of mercury, and the steam is admitted to the still-pot jacket at such a rate that no pressure is built up. The re-boiler section of the column is heated with 15 p.s.i.g. steam and the reflux cooler is turned on full. Only about 2 litres of beer is fed to the column to begin operations in order to avoid foaming, but after distillation has begun the feed valve is re-opened to admit about 8 litres of beer per hour. After 30 litres has been fed in, the feed valve is closed and as much as possible of the water is boiled off, at which time the steam pressure in the still-pot jacket increases and is adjusted to 15 p.s.i.g. Live steam is now sparged into the still-pot at a rate of about 3 litres per hour for three or four hours in order to remove most of the diol from the residual molasses-like liquid. Following this stripping operation the apparatus is shut down, the diol fraction collected from the base of the column, and the distillate and residual liquid discarded.

Experimental Results

The production of diol for laboratory studies was of primary importance and no attempt was made to conduct properly designed experiments covering all conditions of operation. Operation of the unit provided nevertheless an opportunity to acquire data useful in the designing of a pilot plant and some of these data are outlined below.

1. *Cook*

Freedom of the fermenters from contaminants was closely related to the entire absence of dry material or lumps in the cooker. Early in the work with this unit considerable trouble was encountered with contaminants chiefly of the *Clostridium butyricum* type. Efficient mixing of the mash plus pre-liquefaction with malt overcame this difficulty. While pre-liquefaction is advantageous, a marked increase in sugar content may lead to caramelization, especially at cooking pressures above 15 p.s.i.g. This is definitely detrimental to the growth of *Aerobacillus* cultures. A 20 min. malting period at 70° C. with 1% malt (on basis of grain bill) is satisfactory, but as indicated in Table I considerable variation in the malting procedure, and hence in the

TABLE I

EFFECT OF VARIOUS MALTING CONDITIONS ON THE SUGAR CONTENT OF THE COOKED MASH

Malt added, %	Time, min.	Temp., °C.	Sugar, mgm./100 gm. of mash	Appearance of mash
0.5	20	70	307	Slightly viscous
1.0	20	60	666	Good
1.0	20	70	363	Good
1.0	20	75	351*	Good
1.0	10	80	211	Viscous
2.0	20	70	476	Good

sugar content of the mash, is permissible without unduly affecting the fermentability or viscosity of the mash. The liquefying enzyme acts most effectively at 70 to 75° C.; below this temperature the saccharafying enzyme is most active. Above 75° C. the enzymes are inactivated before maximum liquefaction occurs.

Fermentation

The inoculum for the fermenters was developed from fresh agar slants each time by three 24-hr. stages. The initial transfer was made to a 10 ml. test-tube containing a medium made from soluble starch, yeast extract, and calcium carbonate, and the following transfers were made to 200 and 2000 ml. flasks containing a similar medium in which wheat replaced the soluble starch. Modifications of these media were used but their effects on the propagation will be discussed in a later paper in this series. The usual propagation temperature was 30° C. but an interesting variation was the use of 37° C. for the propagation, including the first 8 to 10 hr. in the fermenters themselves. This procedure had the effect of slightly increasing the rate of fermentation, but the results were not as consistent as could be desired and varied with different strains of the organism.

Owing to the comparatively large volumes involved and the simple type of equipment used for fermentation, experimental work involved many difficulties, and laboratory results, presented in other papers of this series, were relied on to a considerable extent. A comparison was, however, made between fermentations in 500 ml. flasks and those in our 5 gal. fermenters, and the data are presented in Table II. Large samples were taken from the cooker and subdivided after thorough mixing, then sterilized for one hour at 15 p.s.i.g. This treatment did not seem to lead to excessive caramelization. In general, there are no differences between these fermentations except that in the fermenters the diol–alcohol ratio is invariably lower. This is readily explained by the more anaerobic conditions in the deeper fermenters.

In general it may be said that N.R.C. Strain C42(3) gave the most consistently satisfactory results in the large fermenters. Only a few of the many

TABLE II

COMPARATIVE DATA FOR FLASK AND LARGE-SCALE FERMENTATIONS ON COOKED MASHES

Cook number	1	2	3	4	5	6
Strain used	C38(2)	C38(2)	C38(2)	C25	C25	C25
Analytical yield						
1. Diol						
(*a*) 5 gal. fermenter	1.60	1.60	1.62	1.55	1.45	1.51
(*b*) 500 ml. flask	1.75	1.82	1.78	1.65	1.65	1.79
2. Alcohol						
(*a*) 5 gal. fermenter	1.22	1.23	1.28	1.23	1.10	1.25
(*b*) 500 ml. flask	1.11	1.17	1.17	1.00	1.03	1.05
3. Total products						
(*a*) 5 gal. fermenter	2.82	2.83	2.90	2.78	2.55	2.76
(*b*) 500 ml. flask	2.86	2.99	2.95	2.65	2.68	2.84
Ratio diol/alcohol						
(*a*) 5 gal. fermenter	1.31	1.30	1.27	1.26	1.32	1.21
(*b*) 500 ml. flask	1.58	1.56	1.52	1.65	1.60	1.70

NOTE: *All flask analyses carried out in duplicate.*

strains available were tried in the plant but of these N.R.C. Strains C38(2), C4(2), C3(3) and C25 and NRRL Strain 510 were satisfactory.

Filtration

The development of a method for the clarification of the raw beer obtained from the fermenters was one of the most difficult problems encountered. Numerous materials were tried in an attempt to coagulate the wheat proteins to a sufficient extent to allow more complete decantation or filtration in a filter press. This object was not attained although good coagulation resulted when the mash was boiled with the following additions:

(1) Brucite to give a pH of 8.5 or higher,

(2) Calcium hydroxide to give a pH of 10 or higher,

(3) Certain dolomitic limestone powders to give pH of 10 or higher.

In addition, ferric chloride, sodium phosphate, and ethyl alcohol were tried at various pH values but without satisfactory results.

The original procedure used with the filter bed involved liming the mash to a pH of 10 and boiling this material, then adding it hot to the filter beds. Although most mashes filtered faster when treated in this way than when unboiled certain ones formed a loose gel that could neither be filtered nor decanted. The conditions favouring this gel formation are not fully understood but the strain of bacteria used for the fermentation affected it; mashes fermented by N.R.C. Strain C2(*a*) invariably behaved in this way.

The filterability of the mashes varies considerably and all the conditions controlling it are not yet understood. Mashes containing starch are always difficult to filter, but even starch-free mashes generally filter more readily if allowed to stand for an additional period. Another interesting

point is that mashes that had been agitated during the course of the fermentation were always more difficult to filter.

Owing to the inefficient washing of the cake, the percentage recovery of diol in this filtration process was seldom as high as was desirable. Data for two operations are presented in Table III but since the mash from each 5 gal. fermenter was filtered separately the data given are the result of three and five filtrations respectively. Although greater volumes of wash water increase

TABLE III

DISTRIBUTION OF THE DIOL IN FILTRATION

	Expt. I	Expt. II
Volume of mash filtered, litres	78.5	158
Volume of wash water used, litres	12.5	20
Total filtrate, including wash water, litres	54.0	135
Weight of wet cake, kgm.	23.2	58.2
Diol in mash, %	1.99	2.21
Diol in filtrate, %	2.05	2.11
Diol in cake, %	0.92	1.37
Diol in cake, % of total in mash	15.0	20.0

the recovery, dilution of the filtrate was undesirable, and in order to avoid this the volume of wash water used was limited to an estimated 50% of the weight of the cake. The wash water was added with the least possible disturbance of the cake so that washing was by displacement.

Distillation

The exact conditions and rate of any distillation are of course dependent upon the column used, but those outlined below were found to be the most desirable under our conditions. There has always been an irregular but unavoidable loss of some diol, presumably due to catalytic decomposition in the presence of iron, and we believe that the lower temperatures possible with vacuum help to keep this loss down. However, a high vacuum with our column caused considerable diol to pass into the distillate.

The advantage of the slow feed, aside from foam control, arose from the fact that by this method a more concentrated diol solution than the original beer was being boiled, and so more diol was carried to the column during the initial part of the operation than would have been the case if the day's run were added as a 30 litre batch. This in turn reduced the stripping time required to complete the removal of the diol. In practice it was found exceedingly difficult to match the feed rate to the distillation rate but even so 40 to 50% of the diol was often removed before stripping began, as compared to about 30% when the beer was added as a 30 litre batch.

The removal of the diol from the residual molasses-like material in the still-pot has been one of the most difficult problems in the recovery process and will be referred to in some detail in a later paper. The steam stripping

method used in this unit is satisfactory for the purposes for which the unit was intended, but can be used efficiently only if a thin layer of material is to be stripped. Because of this the method is uneconomical owing to the relatively large steam consumption and to the length of time the still remains unavailable for the treatment of more clear beer. Table IV presents data showing the recovery of diol in the distillation process. Experiments I and II each represent a single day's operation while for Experiment III composite samples of 13 days' operation were analysed. Three hours' stripping was given to each residue and the distillate volume shown includes the condensate gathered during this period. The over-all recovery for the distillation stage was thus 65 to 70% of the diol present in the clear beer, or 50 to 55% of that shown by the analysis of the fermented mashes.

TABLE IV

DISTRIBUTION OF DIOL IN DISTILLATION

	Volume, litres	Diol, %	Diol, gm.	Diol, % of total
Expt. I				
Feed to still	35	1.37	480	100
Distillate	49	0.08	37	7.7
Residue	5.4	0.48	26	5.4
Diol fraction	1.64	21.5	353	73.5
Loss			64	13.4
Expt. II				
Feed to still	40	1.49	596	100
Distillate	50	0.11	55	9.2
Residue	3.2	1.53	49	8.2
Diol fraction	1.54	25.8	397	66.6
Loss			95	16.0
Expt. III				
Feed to still	374	1.71	6395	100
Distillate	485	0.06	291	4.6
Residue	43	2.60	1118	17.5
Diol fraction	8.5	48.2	4097	64.1
Loss			889	13.8

The diol fraction obtained from the still at the end of the run included all the liquid in the column, so its diol content averaged about 50%. In addition to the diol and water, it contained sufficient impurities to make it turbid and brown and to give it an objectionable odour. Part of these impurities were metallic oxides arising from the iron column but the greater portion were organic products of fermentation or products that were formed by decomposition of more complex material during distillation.

Purification of the Crude Diol

The crude diol obtained from the distillation of the beer required further purification, which could be accomplished by one or two redistillations. A simple distillation under vacuum through an "Eastman" or a similar laboratory column suffices to divide the impure mixture into water, diol, and residual (solid) fractions, but diol obtained in this manner here, while colourless at first, turned dark yellow or brown after standing for a few days. In addition it retained an unpleasant odour. It was therefore necessary to investigate the effect of distillation in the presence of added chemicals and through more efficient columns.

A second distillation of the purified but coloured diol in the presence of excess of either charcoal, bentonite, sodium hydroxide, calcium hydroxide, or calcium oxide was tried but only the calcium salts gave desirable results. Sodium hydroxide pellets served to retain much of the coloured impurity but reacted with the diol to too great an extent. Calcium hydroxide seemed to give as good results as calcium oxide but was more difficult to use, as its powdery nature led to uneven heating in the distillation flask. Lump calcium oxide was therefore used for this purification until such time as Dr. A. C. Neish made a careful study of the fractionation of the crude diol solution. The diol distilled from calcium oxide had a moderate smoky odour and remained colourless for several hours, finally becoming clear yellow.

The present method of purifying the diol is a distillation through a 30 plate Stedman column (3 in. diameter) arranged so that about a 2 : 1 reflux ratio can be maintained. Distillation of 6 litres of 50% diol requires about six hours by this method, the water fraction being removed at about 55° C. with a steadily increasing vacuum during the first 2 to 2½ hours and the diol at about 70° C., with the fullest vacuum obtainable, during the remainder of the time.

At the beginning of the distillation a few drops of a yellow water-soluble compound, probably diacetyl, is obtained. This is followed by a large water fraction, which in the later stages is alkaline with free ammonia. The break between diol and water is relatively sharp but it has been found advantageous to take an intermediate fraction of up to 200 cc. This fraction usually contains much of the colouring matter and will turn deep yellow or even brick red after standing 12 to 24 hr. Little variation in the purity of the diol occurs during the remainder of the distillation unless it is allowed to continue until the accumulation of solids in the still-pot leads to uneven heating and decomposition begins. This is marked by the appearance of smoke in the condenser, and diol collected after this has become evident retains a marked smoky odour and shows more tendency to coloration. The main diol fraction obtained in this manner is almost odourless and even after weeks of storage shows only a lemon yellow tint.

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